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(54) Title: METHODS AND CHEMICAL COMPOUNDS FOR MODIFYING POLYMERS**(57) Abstract**

The invention relates to methods and compositions for improving the fluid, electrical or strength properties of a polymer by binding an effector moiety to the polymer via a protein. The invention particularly relates to improving the properties of paper by binding thereto a moiety capable of conferring a property such as improved wet strength, dry strength or sizing, via a protein such as a cellulase capable of binding to cellulose in the paper.

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METHODS AND CHEMICAL COMPOUNDS FOR MODIFYING POLYMERS**1 Technical Field**

5 The present invention relates to methods and chemical compounds for modifying the physical properties of a polymer. In particular, the present invention relates to methods and chemical compounds for modifying the physical properties of a polymer by binding to the polymer a chemical
10 compound, hereinafter referred to as an "effector moiety", which confers on the polymer improved fluid, electrical or strength properties.

2 Background

15

Polymers and materials containing polymers are a ubiquitous feature of every day life. Naturally occurring polymers include, for example, proteins (including keratin, which is the principal component of wool), starch, pectin, guar,
20 chitin, lignin, agar, alginate, and polysaccharides such as cellulose and hemi-celluloses (including xylan, mannose and arabinose). Cellulose is encountered in the form of, for example, wood fibre and annual crop fibre (for example, hemp, straw, rice, flax, jute) based products such as paper,
25 and cotton, which may be in the form of fibres, yarns, threads or a variety of woven and non-woven textile or fabric products. Xylanose is the principal component of xylan, otherwise known as hemi-cellulose which occurs in grasses, cereal, straw, grain husks and wood. Starch occurs
30 in seeds, fruits, leaves, bulbs etc.

The physical properties of polymers and materials containing polymers may be modified by a variety of chemical and physical treatments. Such chemical and physical treatments
35 may be directed at modification of the polymer structure itself or at modification of the bulk properties of the material containing the polymer.

The bulk properties of a material containing a polymer may, for example, be modified by admixture to the material of agents such as wet strength agents, dry strength agents or other chemical compounds which modify the physical 5 properties of the material. Admixture of such chemical compounds to the material typically does not bind the compounds strongly to the polymer and problems may therefore be experienced with wastage of the chemical compounds and with the compounds leaching out of the material, resulting 10 in variations in the properties of the material. Leaching out of the chemical compound may be reduced by a charge balancing protocol in which the ionic charge of the chemical compound is made equal and opposite to that of the polymer-containing material. However, in practical systems, the 15 charge on both components varies widely requiring careful and frequent control measures. The modifying effect of the chemical compound may also rely on covalent binding to the polymer in order to properly achieve a modifying effect. In addition, promoters may be required to facilitate binding of 20 certain chemicals to the material.

Alternatively, the chemical compounds may be applied to the surface of the material by, for example, immersion or printing. Once again, however, the chemical compounds 25 typically do not bind to the surface of the material and problems may be encountered with diffusion of the compounds away from the intended site of application.

A variety of non-covalent binding interactions are known; 30 for example, the binding interaction between an antibody and an antigen and the binding interaction between biotin and avidin or streptavidin. Enzymes capable of modifying an enzyme substrate also typically rely on a non-covalent binding interaction with the enzyme substrate in order to 35 function.

One such class of enzymes comprise enzymes which degrade polymers, for example proteinases, keratinases, chitinases,

ligninases, agarases, alginases, xylanases, mannases, amylases, cellulases and hemi-cellulases. For example, cellulases and hemi-cellulases cleave saccharide or polysaccharide molecules from cellulose and hemi-cellulose, 5 respectively, and amylases cleave glucose from starch.

The interactions between cellulose and cellulase proteins, in particular those that bind to the cellulose fibres as a prerequisite to catalytic activity have been described and 10 reviewed (cellulase: Béguin, Annu. Rev. Microbiol., 44, 219-248, 1990; cellulases and xylanases: Gilbert and Hazelwood, Journal of General Microbiology, 139, 187-194, 1993). This group of enzymes include cellulases and hemi-cellulases which comprise functionally distinct protein 15 domains. In particular, the domain responsible for catalytic activity is structurally distinct from the cellulose binding domain. These domains are evolutionarily conserved sequences which are very similar in all such proteins (Gilkes et al., Microbiological Reviews, 303-315, 20 June 1991).

The binding domains of such proteins can be separated from the active-site domains by proteolysis. The isolated binding domains have been shown to retain binding 25 capabilities (Van Tilburgh, et al., FEBS Letters, 204(2), 223-227, August 1986). Use of cellulose binding domains of cellulases has been proposed as a means of roughening the texture of the surface of cellulosic support, while use of cellulase active-site domains has been proposed as a means 30 of smoothing the texture of such surfaces (International patent application WO93/05226).

A number of binding domains have also been characterised at the genetic level (Ohmiya et al., Microbial Utilisation of 35 Renewal Resources, 8, 162-181, 1993) and have been subcloned to produce new fusion proteins (Kilburn et al., Published International Patent Application WO90/00609; Ong et al., Enzyme Microb. Technol., 13, 59-65, January 1991; Shoseyov et

al., Published International Patent Application WO94/24158). Some of these fusion proteins have then been used as anchor proteins for specific applications. Such proteins have been used as an aid to protein purification 5 through adhesion of the fusion proteins to cellulosic support materials used in protein purification strategies (Kilburn et al., United States Patent 5,137,819; Greenwood et al., Biotechnology and Bioengineering, 44, 1295-1305, 1994). The ability to immobilize fusion proteins onto 10 cellulosic supports has also been suggested as a means of immobilization for enzyme bioreactors (Ong et al., Bio/Technology, 7, 604-607, June 1989; Le et al. Enzyme Microb. Technol., 16, 496-500, June 1994), and as a means of attaching a chemical "tag" to a cellulosic material 15 (International Patent Application WO93/21331).

3 Summary of the Invention

According to the present invention there is provided a method of treating a polymer to achieve an improvement in at 20 least one property selected from fluid, electrical and strength properties comprising binding an effector moiety to said polymer via a protein linkage for the purpose of achieving said improvement, said effector moiety being different from said protein linkage and said protein linkage 25 being different from said polymer, said effector moiety and said protein linkage being present in an amount effective to achieve said improvement.

It will be appreciated that the polymer may comprise a 30 polymeric molecule or a polymeric material comprising polymeric molecules. Furthermore, reference to an effector moiety and a protein linkage refers to at least one effector moiety and at least one protein linkage, respectively. Accordingly, the present invention encompasses a method of 35 treating a polymer to achieve an improvement in at least one property selected from fluid, electrical and strength properties comprising binding at least one effector moiety to at least one polymer via at least one protein linkage for

the purpose of achieving said improvement, said at least one effector moiety being different from said at least one protein linkage and said at least one protein linkage being different from said at least one polymer, said at least one effector moiety and said at least one protein linkage being present in an amount effective to achieve said improvement.

According to a further aspect of the invention, there is provided a method of treating a polymer to achieve an improvement in at least one property selected from fluid, electrical and strength properties comprising contacting said polymer with an effector moiety and a protein for the purpose of achieving said improvement, said effector moiety being different from said protein and also different from said polymer, and said protein being different from said polymer, and said effector moiety and said protein being present in an amount effective to achieve said improvement. The invention encompasses a method of treating a polymer to achieve an improvement in at least one property selected from fluid, electrical and strength properties comprising contacting at least one polymer with at least one effector moiety and at least one protein for the purpose of achieving said improvement, said at least one effector moiety being different from said at least one protein and also different from said at least one polymer, and said at least one protein being different from said at least one polymer, and said at least one effector moiety and said at least one protein being present in an amount effective to achieve said improvement.

30

According to a further aspect of the present invention there is provided a chemical composition comprising:

35

- a) an effector moiety; and
- b) a protein capable of binding said effector moiety to a polymer;

wherein said effector moiety is different from said protein and wherein said composition is capable of achieving an improvement in at least one property selected from fluid, electrical and strength properties of said polymer.

- 5 The invention further provides composition of matter comprising a polymer to which is bound an effector moiety via a protein linkage, said effector moiety being different from said protein linkage, wherein said effector moiety and said protein linkage are present in an amount effective to
- 10 achieve an improvement in at least one property selected from fluid, electrical and strength properties of said polymer.

According to a further aspect of the invention there is

- 15 provided method of treating paper or the constituent fibres of paper to achieve an improvement in at least one property selected from fluid, electrical and strength properties comprising binding at least one effector moiety to said paper or constituent fibres of paper via at least one
- 20 protein linkage for the purpose of achieving said improvement, said at least one effector moiety being different from said at least one protein linkage and said at least one protein linkage being different from said paper or constituent fibres of paper, and said at least one effector
- 25 moiety and said at least one protein linkage being present in an amount effective to achieve said improvement.

30 4 Detailed Description of the Invention

The present invention provides methods and chemical compounds for modifying the fluid, electrical and/or strength properties of a polymer or material containing a

- 35 polymer by binding to the polymer an effector moiety capable of conferring the desired property.

The term polymer includes reference to materials containing

a polymer. The polymer-containing material may consist exclusively of polymer or may comprise polymer in combination with other components.

- 5 The polymer may comprise any polymer of any number of monomeric units. Preferably, the polymer comprises a naturally occurring polymer or a chemically modified derivative thereof. The naturally occurring polymer may, for example, comprise a protein such as keratin, or a
- 10 polysaccharide such as a starch, pectin, guar, chitin, lignin, agar, alginate. Preferably, the polymer comprises a polysaccharide. The polysaccharide may comprise any polysaccharide, for example, mannose, xylose, cellulose or a hemi-cellulose, preferably cellulose. Materials
- 15 comprising cellulose may comprise wood-fibre or annual crop fibre (for example, hemp, straw, rice, flax, jute) based material, such as paper. Alternatively, the material may comprise cotton in the form of fibre, thread or woven or non-woven textile, fabric or cotton-based paper.
- 20 Preferably, the material comprises paper.

The present invention may be employed to modify any fluid, electrical or strength property of the polymer. Properties of the polymer that may be modified include wet strength and

- 25 dry strength, sizing, hydrophobicity, dye resistance and stain resistance, fluid penetration, oil and water repellency, electrical conductivity and resistance, electrical capacitance, pH and biometallic properties.

- 30 The protein employed in the present invention may comprise any protein capable of binding to the polymer. Preferably, the protein is capable of binding the polymer with a dissociation constant of (K_d) less than $1 \times 10^{-3} M$. As used herein, the term "protein" includes peptide, oligopeptide and polypeptide, as well as protein residues, protein-containing species, chains of amino acids and molecules containing a peptide linkage. Where the context requires (for example, when protein is bonded to another molecule),

reference to a protein means a protein residue. The term "protein linkage" refers to a protein or protein residue via which an effector moiety is bound to a polymer. The protein may comprise a naturally occurring protein, or fragment 5 thereof or modified protein obtainable by chemical modification or synthesis or by expression of a genetically modified gene coding for the protein. As used herein the term "modified protein" includes chemical analogs of proteins capable of binding to a polymer. Examples of 10 proteins capable of binding polymers are well known and include enzymes selected from the group comprising cellulases, hemi-cellulases, mannases, xylanases, proteinases, keratinases, chitinases, ligninases, agarases, alginases and amylases. For example, a variety of 15 cellulases are known which are dependent upon binding to cellulose for their activity. Examples of such cellulases are those isolable from bacterial organisms such as *Cellulomonas fimi* and fungal organisms such as *Trichoderma viride*, *Aspergillus niger*, *Penicillium funiculosum*, 20 *Trichoderma reesei* and *Humicula insolens*, available as commercial preparations from Sigma Chemical Sigma-Aldrich Company Ltd., Novo Nordisk A/S, BDH Ltd., or ICN Biomedicals Ltd. Alternatively, the protein may be produced by recombinant DNA techniques as disclosed in, for example, 25 International Patent application WO94/24158. Cellulases generally comprise a cellulase binding domain and a domain responsible for cellulase activity. The present invention may employ the cellulase as a whole or a fragment thereof capable of binding to cellulose. A cellulase binding domain 30 may be obtained from whole cellulase by treatment with protease(s), such as papain. The present invention may employ an exo-cellulase or an endo-cellulase.

35 Preferably, the protein comprises a naturally occurring enzyme which is capable of binding to the polymer. More preferably, in respect of paper, the catalytic activity is deactivated. The catalytic activity of the enzyme may be deactivated by, for example, attachment of the effector

moiety or cross-linking of the enzyme. Cross-linking of the enzyme may be achieved with any suitable protein cross-linking agent such as a dialdehyde such as glutaraldehyde. Preferably, the protein comprises a deactivated naturally occurring cellulase.

- The effector moiety may be attached to the protein capable of binding to the polymer in any convenient manner. For example, the effector moiety may be covalently bonded directly to the protein, via suitable reactive functional groups in the effector moiety and protein. Recognition of suitable reactive functional groups and, if necessary, their chemical modification to facilitate covalent bonding are within the ability of a person of ordinary skill in the art.
- Examples of covalent bond formation include formation of an amide bond between a carboxyl group and an amine group, by means of carbodiimide or dimethyl formamide activation of the carboxyl group.
- The effector moiety may be attached to any suitable part of the polymer binding protein. The effector moiety may be attached to the polymer binding protein at the N-terminal end of the protein, for example via the N-terminal amino group. Alternatively, it may be attached at the C-terminal end of the protein, for example via the C-terminal carboxyl group. Alternatively, the effector moiety may be attached to the protein via an alternative functional group present, for example, in the amino acid chain of the protein or in a side chain thereof or introduced into the protein for the purpose of attachment to the effector moiety. The effector moiety may, for example, be attached via a thiol group present in cysteine, a hydroxyl group present in serine or threonine, an amino group present in lysine or arginine, an amide group present in asparagine or glutamine, a carboxyl group present in aspartic acid or glutamic acid or an aromatic or heteroaromatic group present in phenylalanine, tyrosine, tryptophan or histidine, or derivatives thereof.

The effector moiety may be attached to the protein via a linker. The linker may, for example, comprise a difunctional molecule capable of reacting with a reactive site of the protein and a reactive site of the effector moiety so as to link the protein and effector moiety. It may be advantageous to include such a linker as a spacer between the protein and effector moiety, so that the two species are sufficiently spaced apart so as not to interfere sterically with each other's activity. A linker may also be advantageous in providing suitable functional group with which to join the effector moiety and protein.

Alternatively, or as part of a linker, the effector moiety may be attached to the protein via a non-covalent binding pair of molecules. Examples of such non-covalent binding pairs of molecules include biotin and avidin, streptavidin or neutralite.

Accordingly, one possibility is that the effector moiety is covalently attached to streptavidin whilst the polymer binding protein is covalently attached to biotin. Combining these components facilitates binding of the streptavidin and biotin portions of each component and hence attachment of the effector moiety to the polymer binding protein. It will be appreciated that the effector-streptavidin component may be mixed with the protein-biotin component either before or after the protein component has been bound to the polymer. It will be further appreciated that alternatively the effector moiety may be covalently attached to biotin, whilst the protein is covalently attached to avidin, streptavidin, or neutralite.

It will be appreciated that more than one type of effector moiety may be attached to the polymer. Two or more types of effector moiety may be used in order to reinforce each other's effect or to provide two or more effects simultaneously.

It will be appreciated that in general the effector moiety may be attached to the polymer binding protein either before or after the polymer binding protein is bound to the polymer. The method of the present invention may comprise

5 contacting a conjugate of the effector moiety and the protein with the polymer, or may comprise contacting the effector moiety with a conjugate of the protein and polymer. Alternatively, attachment of the effector moiety to the protein and attachment of the protein to the polymer may be

10 accomplished *in situ* in a one-step process.

The present invention is not limited as to the precise nature of the manner in which the effector moiety is bound to the protein linkage and the protein linkage is bound to

15 the polymer. Binding may be by means of a chemical bond such as a covalent bond or by means of a non-covalent physical interrelation, tie, association, attraction or affinity.

20 The effector moiety may comprise any moiety capable of conferring a desired physical property. The effector moiety may comprise an atom, molecule or chemical compound or residue thereof capable of conferring the desired physical property. In one embodiment the effector moiety comprises

25 a chemical compound capable of conferring a desired physical property. For example, the agent may comprise a wet strength agent such as an aldehyde eg glutaraldehyde or dialdehyde starch or its cationic derivative, polyamide resin, polyacrylamide copolymer glyoxal, glyoxylated

30 polyacrylamide, polyethyleneimine, polyamine epichlorohydrin polymers, polyamidoamine epichlorohydrin polymers, urea formaldehyde and melamine formaldehyde polymers, synthetic latexes, formaldehyde modified proteins or other polymers used for the purpose of imparting wet strength to paper; a

35 dry strength agent such as starch, anionic or cationic starch, polyacrylamide, amphoteric, cationic or anionic polyacrylamide copolymers, anionic or cationic guar, locust bean gum or anionic or cationic modifications thereof,

polyvinyl alcohol, carboxymethyl cellulose; a sizing agent such as rosin acids including abietic acid, adducted rosin acids including saponified fumaric acid gum rosin adduct, derivatives of rosin acids including tall oil, fatty acids 5 including myristic acid, palmitic acid or stearic acid, other hydrophobic agents including alkenyl succinic anhydride (ASA) or 2-oxetanone compounds such as alkyl or alkynyl ketene dimer or multimer (AKD) or derivatives of ASA or AKD, gum, adducted gum, wood or tall oil rosin, saturated 10 or unsaturated carboxylic acids with linear or branched chain lengths of from about 4 carbon atoms chain length to 30 carbon atoms chain length, alkyl ketene dimers made from such carboxylic acids, alkyl succinic anhydride of chain length from about 4 carbon atoms to about 30 carbon atoms, 15 fully or partially fluorinated carboxylic acids or alkyl ketene dimer derived therefrom, fully or partially fluorinated alkyl succinic anhydride; a dye resistance or stain resistance agent; an oil or water repellent agent such as fluorochemical including a fluorinated fatty acid or 20 fluorinated derivative of ASA or AKD; an agent capable of conferring softness such as an agent capable of disrupting cellulose hydrogen bonding including surfactants, detergents, fatty amides or enzymatic agents such as expansin (McQueen-Mason et al., Proc. Natl. Acad. Sci. USA, 25 91, 6574-6578 (July 1995)); an agent capable of conferring electrical conductivity such as a metal; an agent capable of conferring stiffness; an agent capable of conferring absorbency; an agent capable of conferring hydrophilicity; an agent capable of modifying density; a metallising agent; 30 an agent capable of modifying pH, such as a buffer (for example, to impart resistance to acid degradation).

In another embodiment, the effector moiety may comprise a cross-linking or matrix forming agent or residue thereof, 35 which may itself serve to modify the physical properties of the polymer, or may serve to modify the properties of the protein and hence the physical properties of the polymer, or may serve to entrap a further agent capable of modifying the

- physical properties of the polymer. Preferred examples of cross-linking matrix forming agents comprises dialdehydes, such as glutaraldehyde. Dialdehydes such as glutaraldehyde can for example form a matrix with a cellulase derived protein. The cellulase/glutaraldehyde matrix confers improved wet strength and dry strength on paper, sizes the paper and/or may entrap further agents such as TiO_2 or $CaCO_3$.
- 5 10 An extensive review of compounds useful in papermaking is provided by Roberts et al. (Paper Chemistry, Chapman Hall New York, 1991) the entire contents of which are incorporated herein by reference. This reference particularly reviews retention aids, wet strength additives, 15 dry strength additives, sizing agents and fillers.

As used herein, the term "paper" refers to any material in the form of a coherent sheet or web, comprising an interlaced network of cellulose containing fibres derived 20 from vegetable sources optionally mixed with fibres from vegetable, mineral, animal or synthetic sources in various proportions and optionally mixed with fine particles of inorganic materials such as oxides, carbonates and sulphates of metallic elements in various proportions. The term 25 "paper" includes paperboard which refers to paper when the weight of the paper sheet or web is greater than $200g/m^2$.

Vegetable sources of cellulose include wood, straws, Bagasse, Esparto, Bamboo, Kanaf, Grass, Jute, Ramie, Hemp, 30 Cotton, Flax. The crude vegetable derived cellulose is processed to form pulp, the material from which paper is made, either mechanically, chemically or both. Cellulose containing pulps may be described as mechanical, chemimechanical and chemithermomechanical, semi chemical, 35 high yield chemical, full chemical (see "Pulp and Paper, Chemistry and Chemical Technology", Third Edition, Volume 1 pages 164, 165 edited by James P. Cassay ISBN 0-471-03175-5 (v.1)) according to the method of pulp preparation and

purification.

The effector moiety may be attached to the polymer at any
5 suitable stage in the manufacture and processing of the
polymer or material containing the polymer.

If the effector moiety is to be applied to paper, it may be
attached at the pulp stage or at any stage during the
10 formation of the wet pulp matrix or during the pressing and
rolling of the matrix to form paper. Alternatively, the
effector moiety may be attached to the formed paper product
by immersing the paper in a bath containing the reagents for
attaching the effector moiety or by any suitable spraying,
15 spreading, brushing, coating or printing process.

If the effector moiety is to be attached to cotton, it may
again be attached at any stage in the processing of the
cotton fibre. It may be attached to cotton fibre, thread,
20 yarn or to woven or non-woven cotton fabric or textiles.
The effector moiety may be attached by immersing the
material in a bath containing the reagents for attaching the
effector moiety or by any suitable spraying, spreading,
brushing, coating or printing process.

25 By choosing the point in the manufacture of the polymer or
material containing the polymer at which the effector moiety
is attached, control may be exercised as to whether the
effector moiety is distributed throughout the polymer
30 material or is substantially restricted to the surface
levels of the material.

In cases where the effector moiety is directed at modifying
the bulk properties of the material, it may be advantageous
35 to ensure even distribution of the effector moiety uniformly
throughout the material. Accordingly, the effector moiety
should be attached at an early stage in the manufacture. For
example in the manufacture of paper where the effector

moiety is directed at modifying the bulk properties of the paper, the effector moiety should be applied at the pulp stage.

- 5 In cases where the effector moiety is directed at modifying the surface properties of the material, it may be sufficient to restrict the effector moiety to the surface levels of the material, with an attendant advantage in reducing the quantities of effector moiety required. Accordingly, the
- 10 effector moiety should preferably be supplied at a late stage in the manufacture. For example, in the manufacture of paper, where the effector moiety is directed at modifying the surface properties of the paper the effector moiety should be applied to the paper surface.

15

- Depending on the application it may be desirable to apply the effector moiety to one or both planar surfaces of the paper. Treating both surfaces of the paper with for example an effector moiety comprising a wet strength agent, whilst leaving one or more of the edges untreated, facilitates preparation of a sandwich structure, in which a layer of paper having poor wet strength properties but good liquid absorption properties is sandwiched between two layers of paper having good wet strength properties. Such a structure is capable of transporting liquids through its middle layer by capillary action and is particularly useful in the manufacture of dip-stick type diagnostic assays.

- A particular feature of the present invention concerns the
- 30 ability to modify the physical properties of the polymer or material containing the polymer in a reversible manner. Conventional treatment of polymers to impart particular physical properties are often non-reversible. Furthermore, the conventional treatments often render the polymer
 - 35 unsuitable for recycling. In connection with recycling paper, the repulping of paper is made more difficult and may be impossible if the paper is treated with conventional wet strength agents. The present invention lends itself to the

provision of means to permit release of the effector moiety to permit recycling of the material. The effector moiety may, for example, be released from the polymer-containing material by treatment with a protease which cleaves the 5 protein attaching the effector moiety to the polymer; alternatively, the effector moiety may be attached to the protein by means of a selectively cleavable linker; cross-linking agents such as aldehyde-substituted starch may be cleaved by amylase.

10

A further advantage of the present invention lies in the fact that the desired physical property is imparted essentially immediately to the material. In conventional treatments to impart wet strength to paper, heat treatment 15 and curing over several weeks may be required.

The invention will now be described with reference to the following figures and examples. In the Figures:

20 Figure 1 shows the effect of cellulase concentration on glutaraldehyde cross-linked cellulase imparted wet strength.

Figure 2 shows the effect of glutaraldehyde concentration on glutaraldehyde cross-linked cellulase imparted wet strength;
25

Figure 3 shows the effect of pH on glutaraldehyde cross-linked cellulase imparted wet strength;

30 Figure 4 shows the effect of temperature on glutaraldehyde cross-linked cellulase imparted wet strength;

Figure 5 shows the effect of incubation time on glutaraldehyde cross-linked cellulase imparted wet strength;

35 Figure 6 shows the effect of pre-incubation time on glutaraldehyde cross-linked cellulase imparted wet strength;

Figure 7 shows the effect of glutaraldehyde cross-linked

cellulase on the wet strength of paper produced from different wood pulps.

It will be appreciated that the following is by way of example only and modification of detail may be made within the scope of the invention.

EXPERIMENTAL

10 Principles and Applications of Effector Moiety Attachment

The protocols defined below represent the techniques used to characterize the use of cellulase as a *biobridging* agent for the attachment of effector moieties to cellulose.

15 For initial stock preparation one-third strength Phosphate Buffered Saline ($\frac{1}{3}$ PBS) was used. The formulation for $\frac{1}{3}$ PBS was as follows:

20 200 litres of deionized or demineralized water
 (DEMI water)
 197g of anhydrous sodium dihydrogen phosphate
 (NaH_2PO_4)
 767g of anhydrous disodium hydrogen phosphate
25 (Na_2HPO_4)
 389g of sodium chloride (NaCl)

Anhydrous materials are not essential but the above mentioned weights should be recalculated to take into account any "water of crystallization" in the hydrated salts.

The cellulases that have been used were derived from fungal sources and are available either as aqueous solutions or
35 freeze dried powders.

Penicillium funiculosum

Cellulase derived from *Penicillium funiculosum* (Sigma

Aldrich Co. Ltd., Poole, Dorset, U.K.) is available as a tan powder and should be stored at below 0 C.

When used as an additive for handsheets the cellulase was
5 first be prepared as a 20% total solids solution in $\frac{1}{3}$ PBS. Into a large shallow beaker was placed 200g of the dry enzyme preparation. To this was then added slowly 800g of $\frac{1}{3}$ PBS. The mixture was gently stirred with a glass rod. Vigorous agitation of the solution should NOT be used to
10 disperse the powder as denaturing of the enzyme may occur. Any clumps of enzyme preparation may be broken up gently with the glass rod. If the cellulase solution is prepared the day before use then it should be stored at 4 C.

15 Trichoderma Reesei

Cellulase derived from Trichoderma Reesei is available either as freeze dried powder from Sigma Aldrich Co. Ltd. Poole, Dorset, U.K. or as an aqueous solution from Novo Nordisk A/S, Bagsvaerd, Denmark. When using the powder, the
20 procedure and handling practises for preparation of the aqueous solution with Penicillium funiculosum apply here as well.

The cellulase solution was added to the stock on the basis
25 of the total protein content of the enzyme solution (e.g. 10 parts of dry protein per 100 parts of dry fibre). The total protein content of the prepared cellulase solution was determined by the UV absorbance ($\lambda = 620\text{nm}$) of the protein stained with Coomassie Brilliant Blue G250 dye (Sedmak and
30 Grassberg (Analytical Biochemistry, 79, 544-552 (1977)).

1. To assay for the binding of the cellulase to cellulose

Samples (typically between 25 to 500 mg and normally 100 mg)
35 of cellulose, such as microcrystalline cellulose (Avicel, SigmaCell) or water-leaf paper pulp, were weighed into a series of tubes/flasks.

Cellulase solutions (typically containing between 200-600 mg protein ml⁻¹ in 3 ml buffer), were added to each tube. The exact concentration of protein added initially was experimentally determined at the start of the binding assay 5 using the assay developed by Sedmak and Grassberg (Analytical Biochemistry, 79, 544-552 (1977)).

The tubes were shaken at the desired temperature (typically between 4°C and 30°C but usually at room temperature), for 10 a period of time (typically 1 to 90 min, usually between 5 to 15 min). Samples (0.5 - 1ml) were then taken for assay.

The samples were centrifuged in a 1 ml Eppendorf tube using a bench-top microfuge for 5 min and the supernatant retained 15 for determination of protein concentration remaining in the supernatant (unbound cellulase).

The supernatant protein concentration was subtracted from the initial protein concentration thereby defining the 20 amount of cellulase associated with the cellulose pellet.

Bovine serum albumin (BSA) was used in the assay as a control.

25 The results were presented as either the amount of protein bound to the cellulose as a percentage of the protein added, or as the amount of protein bound to the cellulose as a percentage of the protein/cellulose (%w/w).

30 2. Visualization of the effector moiety attachment using chemiluminescence

Preparation of cellulase for the ECL detection system

35 1. Biotinylation of cellulase

A solution of biotinamido N-hydrosuccinimide ester ($B_{cap}NHS$) in N,N-dimethylformamide (DMF) was prepared (1

mg ml⁻¹). A solution of cellulase was prepared in distilled water (77 mg ml⁻¹).

5 1ml of the cellulase solution was added to 1ml of the B_{cap}NHS solution and the mixture incubated for 2.5 h at room temperature with shaking. The reaction was then exhaustively dialysed against 500 ml 1/3 PBS buffer (PBS, pH 7.5: Na₂HPO₄, 11.5g; NaH₂PO₄, 2.96g; NaCl, 5.84 g diluted to 1L with distilled water) for 1 h.

10 2. Binding of the Biotinylated cellulase to paper sheets [Application of the cellulase to the surface of a paper sheet]

15 A water-leaf paper sheet, usually 2 cm², was incubated with biotinylated cellulase at a range of concentrations between 0.05 to 100 µg ml⁻¹ protein in 1/3 PBS (10 ml) for 45 min to 2 h at 4°C in a shallow Petri-dish with shaking. Experiments using PBS containing Tween 20

20 (0.1% vv⁻¹) were also performed.

25 3. Binding of the biotinylated cellulase to paper pulp and subsequent production of a paper sheet [Application of the cellulase to the paper matrix]

30 Paper pulp was incubated with the biotinylated cellulase in 1/3 PBS containing Tween 20 (0.1% vv⁻¹) for 45 min at room temperature with shaking. A disc of paper was formed from the paper pulp-biotinylated cellulase using the paper making filter. The paper disc was removed from the filter, rolled and allowed to dry overnight.

35 4. Binding of HRP-labelled streptavidin to the biotinylated cellulase

Binding of HRP-labelled streptavidin and ECL detection of the biotinylated cellulase was subsequently performed according to the manufacturer's recommendations

(Amersham Ltd., Amersham, U.K.; Whitehead, T.P. et al., Clin. Chem. 26, 1531-1546, 1979).

5 The paper was incubated with milk powder ($4\% \text{ wv}^{-1}$) in PBS for 45 min at either 4°C or room temperature with shaking to block non-specific binding of the HRP-streptavidin conjugate. The paper was then washed, 3x 3 min, using $0.5\% \text{ (wv}^{-1}\text{)}$ milk powder in $1/3$ PBS containing Tween 20 ($0.1\%\text{vv}^{-1}$). The solution was
10 discarded and replaced after each wash.

15 The horseradish peroxidase (HRP) - streptavidin conjugate was prepared as a 1:1000 part solution using milk powder ($0.5\% \text{ wv}^{-1}$) made up in $1/3$ PBS containing Tween 20 ($0.1\%\text{vv}^{-1}$). A suitable volume (2 to 10 ml) was added to cover the paper sheet which was then incubated for 45 min at room temperature with shaking.

20 The paper was then washed 3x 5 min, in $1/3$ PBS containing milk powder ($0.5\% \text{ wv}^{-1}$) and Tween 20 ($0.1\%\text{vv}^{-1}$). The wash solution was discarded and replaced after each wash. The paper was then washed 3x 5 min using $1/3$ PBS and again the wash solution was discarded and replaced after each wash.
25

The cellulose bound cellulase-biotin-HRP-streptavidin conjugate was then visualised by the ECL method or quantified using the OPD methodology.

30 3. Enhanced chemiluminescence (ECL) method

It is necessary to carry out this method in a photographic darkroom.

35 Amersham ECL Detection Reagents 1 + 2 were mixed together in equal volumes (required approximately 0.13 ml cm^2 paper). Excess buffer was then drained from the paper and the

detection reagents added to completely cover the paper surface.

The paper was incubated for exactly 1 min at room
5 temperature without agitation. The detection reagent was drained off and the paper was blotted between two pieces of tissue paper to remove excess reagent. The blotted paper was then transferred to a piece of cling film and wrapped securely to remove any air pockets.

10

The paper was placed in a film cassette minimising the delay between incubating the paper and exposing it to the Hyperfilm. The film was carefully placed on top of the paper and the film exposed for 15 s ensuring that the film
15 did not move during exposure. This first sheet of film was then removed and immediately replaced with a second film which was then exposed for 1 min.

The films were then immediately developed to visualize the
20 results. If necessary further sheets of film can be exposed with exposures of 1 to 60 min.

4. The (OPD) method for quantification of effector moiety bound to cellulose

25

The substrate buffer was prepared by dissolving 1 OPD tablet (60 mg; o-phenylenediamine dihydrochloride, Sigma Chemicals, UK) in 150 ml 0.06 M phosphate-citrate buffer (0.2 M Na₂HPO₄, 121.5 ml; 0.1 M citric acid 121.5 ml made up to 500 ml distilled water and the pH adjusted to 5.0) to give a final OPD concentration of 0.4 mg ml⁻¹. Note that this reagent is light sensitive. 10 µl of fresh 30% H₂O₂ per 25 ml of substrate buffer was added immediately prior to use.

35 The paper samples containing the biotinylated cellulase were placed into a 50 ml Falcon tube. 25 ml of the complete substrate buffer solution was added to the tube and shaken at room temperature for 30 s to 20 min, and usually between

5 and 15 min, then the reaction was stopped by adding 1 ml of 3M H₂SO₄. The absorbence was then determined at 492 nm and reference was made to a standard curve of OD₄₉₂ vs biotinylated cellulase concentration in order to calculate 5 the concentration of biotinylated cellulase present on or in the paper.

5. The coupling of paper effector moieties to enzyme peptides using carbodiimide

10 Carbodiimides react with carboxylate groups to form activated carboxyls. Amino groups then attack these activated carboxyls to form covalent peptide bonds. This chemistry can be used to attach paper effector chemicals 15 which contain free carboxyl groups to the amino groups on peptides.

The carbodiimide chemistry used in the linkage of a paper effect chemical to the cellulase was based on conventional 20 methodology (Hoare et al., J. Biol. Chem., 242(10), 2447-2453, 1967).

In the method described below, abietic acid was coupled to cellulase.

25 Cellulase (21 mg ml⁻¹) was dissolved in distilled water, and abietic acid (100 mg) was dissolved in 25 ml of 10% (vv⁻¹) methanol. 0.5 ml 1-(3-dimethylamino propyl)-3-ethyl carbodiimide-HCl (WS-CDI; 63 mg ml⁻¹) was added to 1.0 ml 30 abietic acid solution and the pH adjusted to pH 4.5 ± 0.5 using HCl (0.1 N). The mixture was then stirred at room temperature (5 min). 2 ml cellulase solution was then added and the mixture left at room temperature with stirring (16 h).

35 The reaction was then stopped by the addition of sodium acetate (0.1 M; pH 5.0) and excess abietic acid and WS-CDI was removed by exhaustive dialysis in phosphate buffer.

The coupled cellulase was then used to bridge the abietic acid onto cellulose as described above.

5 6. Demonstration of wet tensile strength properties using glutaraldehyde cross-linked cellulase.

The application of glutaraldehyde cross-linked cellulase to water-leaf paper pulp has been performed and demonstrated to 10 impart wet-strength properties to the paper sheet.

Water-leaf paper pulp slurry was produced in the following manner: 10 g water-leaf paper was cut into 1 cm² squares and macerated in a domestic herb mill (CH100, Kenwood Ltd. UK) 15 for 3 min with 100 ml distilled water.

2.15 g of a water-leaf paper pulp slurry (10% wv⁻¹) containing 0.2 g cellulose was taken and the following additions were made:

20

- 1 10 ml of 1/3 strength phosphate buffered saline (PBS), pH 7.0 as a negative control.
- 2 10 ml of 1/3 PBS containing *T. reesei* cellulase (2 mg ml⁻¹)
- 25 3 10 ml of 1/3 PBS containing glutaraldehyde (25 µl ml⁻¹)
- 4a 10 ml of 1/3 PBS containing *T. reesei* (2 mg ml⁻¹) and glutaraldehyde (25 µl ml⁻¹) incubated together for 1 h at room temperature prior to addition to the pulp
- 4b 10 ml of 1/3 PBS containing *T. reesei* cellulase (2 mg 30 ml⁻¹) and glutaraldehyde (25 µl ml⁻¹) added directly to the pulp

All the samples were then incubated for 1 h at room temperature on an orbital shaker before production of the 35 paper sheets.

To produce the paper sheets, the volume was increased to 100 ml with distilled water and paper sheets (6 cm²) produced

using a laboratory-designed paper making apparatus operated in the following manner: a suspension of paper pulp (0.2% wv⁻¹) was poured into a plastic filter holder which houses a fine nylon filter mesh. By applying a vacuum for a few 5 seconds the pulp was formed into a paper sheet supported by the mesh. The filter mesh was removed from the apparatus and the paper sheet sandwiched between a second nylon mesh and blotted between adsorbent paper towels. The paper sheet was carefully removed from the paper-making mesh, flattened 10 by rolling and allowed to dry overnight.

Wet-strength was determined in the following ways;

A Paper stability in water

15 Samples from each test paper sheet (1.5 cm²) were placed in Universal bottles and 25 ml distilled water added to each one. The tubes were shaken and periodically examined for signs of loss of integrity of the paper samples.

20 The results are given in Table 1

Table 1 determination of the stability of the paper samples in water

| 5 | Sample Number | Replicate Number | Condition after shaking | Initial Disruption | Total Disruption |
|----|---------------|------------------|-------------------------|--------------------|------------------|
| 10 | 1 | 1 | Disintegrated | - | - |
| | | 2 | Disintegrated | - | - |
| 15 | 2 | 1 | Disintegrated | - | - |
| | | 2 | Disintegrated | - | - |
| 20 | 3 | 1 | Disintegrated | - | - |
| | | 2 | Disintegrated | - | - |
| 25 | 4a | 1 | Intact | <18 h | 36 h |
| | | 2 | Intact | <18 h | 36 h |
| 25 | 4b | 1 | Intact | >8 d | >8 d |
| | | 2 | Intact | >8 d | >8 d |

In Table 1, "--" means not applicable.

30 B Paper Strength

Samples from each test paper sheet (4 cm x 1 cm) were taken and 25 µl of distilled water was pipetted across the middle of the paper ensuring an even distribution. The paper was suspended between two bulldog clips and a container was secured to the bottom clip. Water was added to the container and the weight of water necessary to cause the paper to tear was determined.

The results are given in Table 2 and illustrate that the samples prepared using glutaraldehyde cross-linked cellulase demonstrated the greatest wet tensile strength.

Table 2 determination of paper strength

| 45 | Sample Number | Replicate Number | Added Weight (g) |
|----|---------------|------------------|------------------|
| | | | |

| | | |
|-----|---|--------|
| 1 | 1 | 27.43 |
| | 2 | 43.48 |
| 5 2 | 1 | <22.00 |
| | 2 | <22.00 |
| 3 | 1 | <22.00 |
| | 2 | 34.63 |
| 10 | | |
| 4a | 1 | 66.34 |
| | 2 | 49.96 |
| 4b | 1 | >77.33 |
| 15 | 2 | 64.20 |

The wet-strength of the paper samples were retested to include BSA controls to assess the specificity of action of the bridging protein. The paper samples were prepared as follows

- 20 1 10 ml of 1/3 strength phosphate buffered saline (PBS), pH7.0
- 2 2 10 ml of 1/3 PBS containing *T. reesei* cellulase (2 mg ml^{-1})
- 3 3 10 ml of 1/3 PBS containing glutaraldehyde ($25 \mu\text{l ml}^{-1}$)
- 4 4 10 ml of 1/3 PBS containing BSA (2 mg ml^{-1})
- 25 5 10 ml of 1/3 PBS containing *T. reesei* cellulase (2 mg ml^{-1}) and glutaraldehyde ($25 \mu\text{l ml}^{-1}$) added to the pulp and incubated for 1 h at room temperature on an orbital shaker before production of the paper sheets
- 6 6 10 ml of 1/3 PBS containing BSA (2 mg ml^{-1}) and glutaraldehyde ($25 \mu\text{l ml}^{-1}$) added to the pulp and incubated for 1 h at room temperature on an orbital shaker before production of the paper sheets.

35 The paper samples were placed in 50 ml Universal bottles with 25 ml water and vortexed using a laboratory mixer until complete disintegration of the paper samples occurred. The results are given in Table 3.

Table 3 Determination of the stability of the paper when vortex mixed in water.

| 5 | Sample Number | Replicate Number | Time required until complete disintegration (sec) |
|----|---------------|------------------|---|
| | 1 | 1 | <5 |
| 10 | | 2 | <5 |
| | 2 | 1 | <5 |
| | | 2 | <5 |
| 15 | 3 | 1 | <5 |
| | | 2 | <5 |
| | 4 | 1 | <5 |
| | | 2 | <5 |
| 20 | | | |
| | 5 | 1 | >1020 |
| | | 2 | >1080 |
| | 6 | 1 | 10 |
| 25 | | 2 | 10 |

Although samples prepared using cross-linked BSA (Sample 6) showed an increased wet tensile strength compared to the 30 controls, this was 100 fold less than that of the glutaraldehyde cross-linked cellulase.

To determine optimum conditions for glutaraldehyde/cellulase treatment of paper to improve wet-strength, the following 35 parameters were varied in turn. For the purposes of this work the control parameters were set at: cellulase (2 mg ml^{-1}); glutaraldehyde ($0.6\% \text{ vv}^{-1}$) added separately to the pulp; pulp suspended in buffer (pH 7.0) at 25°C . The mix was incubated

for 60 min before paper sheet formation. Each parameter was varied in turn as follows: cellulase (0.5 to 8 mg ml⁻¹); glutaraldehyde (0.1 to 2.5 vv⁻¹); pH (5.0 to 10.0); temperature 25°, 37° and 45°C; incubation time (5 to 120 min); and time of 5 pre-incubation of the cellulase and glutaraldehyde (15 to 60 min).

All the paper sheets were allowed to dry overnight at ambient temperature prior to wet tensile strength testing. The results 10 are illustrated in Figure 1 to 6.

7. Demonstration of Glutaraldehyde Cross-linked Cellulase (GCC)-Wet Tensile Strength Properties with Different Pulp 15 Types.

The GCC wet strength composition was applied to paper produced from different types of pulp: ground wood pulp (GWP), chemo-thermo-mechanical pulp (CTMP), hard wood pulp (HWP), soft wood 20 pulp (SWP) and water-leaf pulp (W-LP; 70% HW: 30% SW). The pulps were prepared in the usual manner, however, GWP and CTMP pulps were soaked in water overnight before blending to promote dispersion of the fibres.

25 The pulps were treated with either PBS buffer (10 ml); PBS buffer (10 ml) + cellulase (20 mg) + glutaraldehyde (0.6% vv⁻¹). Then paper sheets (6 cm²) were prepared from the pulp samples as described above before wet tensile strength testing. The results are given in Table 4 and illustrated graphically 30 in Figure 7.

The results indicate that there was an improvement in the wet tensile strength of all the pulps tested. The final strength of the paper sheets produced using either GWP or CTMP was 35 greater than that of the HWP, SWP and W-LP. The GCC composition did however induce a greater percentage increase in tensile strength in the HWP and SWP samples.

Table 4: Wet Tensile Strength of Paper Sheets Produced from Different Pulps.

| Pulp | Treatment | Vortex mixing ¹ (seconds) | Wet Tensile Strength (g) |
|-------------|----------------------------|---|--------------------------------|
| 5 | | | |
| Ground wood | Buffer | 135 | 105.4 |
| | Cellulase | 120 | 45.0 |
| 10 | Glutaraldehyde | 315 | 94.5 |
| | Cellulase + Glutaraldehyde | > 1200 | 249 |
| 15 | | | |
| CTMP | Buffer | 480 | 78.5 |
| | Cellulase | 420 | 88 |
| | Glutaraldehyde | 420 | 71.5 |
| | Cellulase + Glutaraldehyde | > 1200 | 242.5 |
| 20 | | | |
| HWP | Buffer | < 5 | < 15.3 |
| | Cellulase | < 10 | < 15.3 |
| | Glutaraldehyde | < 10 | < 15.3 |
| | Cellulase + Glutaraldehyde | > 300 | 119 |
| 25 | | | |
| SWP | Buffer | < 5 | < 15.3 |
| | Cellulase | < 5 | < 15.3 |
| | Glutaraldehyde | < 5 | < 15.3 |
| | Cellulase + Glutaraldehyde | > 300 | 207 |
| 30 | Glutaraldehyde | | |
| 35 | | | |
| W-LP | Buffer | < 5 | < 15.3 |
| | Cellulase | ND | ND |
| | Glutaraldehyde | ND | ND |
| | Cellulase + Glutaraldehyde | > 300 | 193 |

¹ Time needed to complete disruption

8. Demonstration of the reversibility of glutaraldehyde cross-linked cellulase imparted wet tensile strength

To determine the reversibility of wet strength imparted by the
5 glutaraldehyde cross-linked cellulase the following protease
solutions were prepared using commercial protease preparation
supplied by Sigma Chemical Sigma-Aldrich Company Ltd., Fancy
Road, Poole, Dorset, BH17 7NH: ficin ($4\mu\text{l ml}^{-1}$ solution in PBS
buffer at pH 6.5); papain ($5\mu\text{l ml}^{-1}$ solution in PBS buffer at
10 pH 6.5); Protease K (2.8 mg ml^{-1} solution in PBS buffer pH
8.0); α -chymotrypsin (1.0 mg ml^{-1} solution in PBS buffer at pH
8.0).

Paper squares ($1.5 \times 1.5 \text{ cm}$) prepared from water-leaf paper
15 pulp strengthened with glutaraldehyde cross-linked cellulase
were taken and incubated with the following treatments outlined
in Table 5.

Table 5 Wet strength reversibility treatments

| Sample No. | Treatment |
|------------|---|
| 5 1 | Ficin (10 ml) + PBS buffer (pH 6.5) |
| 2 | Papain (10 ml) + 10 ml PBS buffer (pH 6.5) |
| 3 | Protease K (1 ml) + 19 ml PBS buffer (pH 8.0) |
| 10 4 | α -chymotrypsin (1 ml) + 19 ml PBS buffer (pH 8.0) |
| 5 | Ficin (10 ml) + Papain (10 ml) |
| 15 6 | Protease K (1 ml) + α -chymotrypsin (1 ml) + 18 ml PBS buffer (pH 8.0) |
| 7 | Ficin (10 ml) + Papain (10 ml) + Protease K (1 ml) + α -chymotrypsin (1 ml) |
| 20 8 | 0.2 M Phosphate buffer pH 6.5 (20 ml) |
| 9 | 0.2 M Phosphate buffer pH 8.0 (20 ml) |
| 25 | The samples were incubated at 30°C on an orbital shaker at 70 rpm. The samples were examined after 4h and 20h and were vortex mixed for 10 sec after 20 h if the paper was still intact. The determinations were performed in duplicate and the results are given in Table 6. |
| 30 | |

Table 6 determination of paper disruption in the presence of various treatments

| | Treatment | Incubation Time (h) | | After further 10 sec vortex |
|----|--|----------------------------|-----------|--|
| | | 4 | 20 | |
| 5 | | | | |
| | Ficin | X | XX | XXXX |
| | | X | XX | XXXX |
| 10 | Papain | XX | XXXX | --- |
| | | XX | XXXX | --- |
| | Protease K | XX | XX | XXXX |
| | | XX | XX | XXXX |
| 15 | | | | |
| | α-chymotrypsin | 0 | XX | XXXX |
| | | 0 | XX | XXXX |
| | Papain + Ficin | XX | XXXX | --- |
| 20 | | XX | XXXX | --- |
| | α-chym + Prot K | X | XX | XXXX |
| | | X | XX | XXXX |
| 25 | All 4 proteases | XX | XXXX | --- |
| | | XX | XXX | XXXX |
| | Control | 0 | 0 | 0 |
| | Phosphate | 0 | 0 | 0 |
| 30 | buffer (pH 6.5) | 0 | 0 | 0 |
| | Control | 0 | 0 | 0 |
| | Phosphate | | | |
| | buffer (pH 8.0) | | | |
| 35 | | | | |

The key to the qualitative observations is given as an arbitrary scale of 0 to XXXX where 0 represents no visible

disruption of the paper and XXXX represents total paper disruption; ---- represents previous loss of paper integrity.

9. To determine the effect of protease treatment on the
5 recyclability of paper wet strengthened by glutaraldehyde
cross-linked cellulase

Paper sheets prepared either from water-leaf paper pulp (0.2 g) wet strengthened with glutaraldehyde cross-linked cellulase
10 or with pulp (0.2 g) prepared without any wet strength agent were taken and subjected to a series of treatments.

Treatment 1 A paper sheet (0.2 g) made from pulp wet strengthened with glutaraldehyde cross-linked cellulase was cut
15 into 1 cm x 1 cm squares which were placed in a petri-dish with 20 ml 0.2 M phosphate buffer (pH 8.0) containing Protease K (14 mg). The sample was incubated at 37°C for 2 h with shaking (60 rpm). The squares were then removed and dipped into phosphate buffer (pH 8.0) and placed in a universal bottle containing 20 ml fresh phosphate buffer (pH 8.0). The sample was then vortex mixed to macerate the paper. Any fibres left in the petri-dishes after the original incubation were harvested by centrifugation at 6,000 rpm, washed with phosphate buffer (pH 8.0) and added to the macerated sample in the universal bottle.
25 2 ml *T. reesei* cellulase (10 mg ml⁻¹) and 0.5 ml glutaraldehyde solution (25%) were added and the sample was incubated at 25°C for 60 min. The sample was then used to form a new sheet of paper.

30 Treatment 2 A water-leaf paper sheet (0.2 g) made from pulp prepared with PBS without any wet strength agent was placed in a universal bottle with 14 ml 1/3 strength PBS. The sample was vortex mixed to macerated the paper and the pulp was made into a fresh piece of paper.

35

Treatment 3 Squares of paper made from pulp wet strengthened with glutaraldehyde cross-linked cellulase (0.4 g) were macerated in a blender with 30 ml of 1/3 strength PBS.

The resultant pulp was removed and made into a fresh square of paper.

Treatment 4 A glutaraldehyde cross-linked cellulase strengthened paper sheet (0.2g) was cut into 1 cm² pieces and macerated in a blender in 30 ml of 1/3 strength PBS. 20 mg *T. reesei* cellulase (10 mg ml⁻¹) and 0.5 ml glutaraldehyde solution (25% vv⁻¹) were added. The sample was incubated on an orbital shaker for 60 min at room temperature. The pulp was used to prepare a new sheet of paper.

Each paper sheet was allowed to dry overnight at ambient temperature before testing the integrity of 1 cm² samples to destruction in water using the vortex mixer. The results of the paper determination are given in Table 7.

Table 7: Integrity of Recycled Paper

| Strategy | Vortex mix (Time, s) | Description of paper |
|----------|-------------------------|-------------------------------|
| 1 | 300 | Paper broken into 3 pieces |
| 2 | 5 | Paper totally disintegrated |
| 3 | 50 | Lots of small fragments |
| 4 | 345 | Small hole in middle of paper |

These results indicate that GCC - containing pulp, when made into a new paper sheet, retains some wet tensile strength properties; that pulp produced by protease treatment, as opposed to physical disruption, generates stronger paper when

recycled and that the further addition of GCC imparts the best wet tensile strength properties to the recycled sheets.

10. Demonstration wet-strength, dry-strength and sizing in
5 paper following treatment with glutaraldehyde and
cellulase

10 Experiments were performed to determine the effect of cellulase (protein ligand) and glutaraldehyde (effector moiety) on the wet strength, dry strength and sizing of paper. In the experiments, the following materials and general protocols were employed:-

Cellulase

15 An aqueous *Trichoderma reesei* cellulase preparation was employed ("Cellulast 1.5L" supplied by Novo Nordisk Bioindustry S.A. 92017 Nanterre Cedex. France)

Glutaraldehyde

20 The glutaraldehyde used in the following examples was a 25% aqueous solution commercially available from Merck Ltd. (Poole, Dorset, U.K.)

Stock Preparation

25 Except where otherwise indicated, the furnish used was a blend of ECF bleached hardwood and softwood pulps (ratio of 70:30 HW/SW). The stock was prepared with $\frac{1}{3}$ PBS and no fillers were added. The procedure was as follows:

30 280g of bleached hardwood pulp and 120g of bleached softwood pulp were added to 18 litres of $\frac{1}{3}$ PBS. The fibres were dispersed by vigorous agitation. This stock was then transferred to the Hollander and beaten until a freeness value of 25oSR was attained (time taken was usually 30 to 35 minutes). The stock was then adjusted to a final consistency of 2% with further $\frac{1}{3}$ PBS as necessary.

Addition/Incubation of Additives

Both the cellulase solution and glutaraldehyde solution were added to the thick (2% consistency) stock. Two litres of the thick stock (containing 40g of fibre) was contained in a metal jug and stirred at the lowest possible speed to achieve a slow movement of the stock. Vigorous agitation should be avoided otherwise denaturing of the enzyme may occur during the incubation period. The stock was at ambient temperatures (20-25°C). The cellulase solution was added first to the stock (avoiding any splashing or splattering of the solution). When one minute had elapsed from the addition of the enzyme, the aqueous glutaraldehyde was added.

The incubation time of the additives was fifteen minutes, starting from the end of enzyme addition. During this incubation period the movement of the stock may appear to become easier/faster. If this is apparent then reduce the stirrer speed as much as possible.

After the fifteen minute incubation period had elapsed the thick stock was then added to the proportioner.

Proportioner

The thick stock in the proportioner was then diluted to a consistency of 0.25% using DEMI water only. Normal agitation speeds in the proportioner were employed to mix the stock.

Handsheet Formation

The white water box was filled with DEMI water for handsheet formation. With the handsheet forming wire in place in the mould assembly, one litre of stock from the proportioner was added to the Deckle

5 Box, together with water from the white water box. The contents of the Deckle Box were agitated with the perforated agitator (moved up and down five times). After the fifth stroke the agitator was rested on the surface of the water to help dampen the motion of the water in the Deckle Box. The water was then pumped back to the white water box and the initial wet mat was formed.

10 Depending on how vigorous the agitation has been some foaming may occur in the Deckle Box. This foam may still persist after the initial wet mat is formed and can be quite substantial. Some of this foam can be dispersed if the pump is kept on for a few seconds after the water has been removed so that air can be drawn through the mat.

15

20 Handsheet Pressing and Drying
The wet mat and handsheet wire were removed from the mould to the press. The moisture content of the pressed sheet should be 70%. The pressed sheet was then dried on an electrically heated drum dryer. The surface temperature of the dryer was between 60°C and 105°C and the speed of the dryer was such that the pressed sheet was in contact with the hot surface for 35 to 180 seconds. The final moisture content of the sheet should be between 4 and 7% (typically 5%).

25
30 If the moisture content of the sheet after pressing is less than 70%, then the sheet may stick to the surface of the drum dryer when the above conditions are employed. This may occur because of nonuniform press pressures being applied across the width of the sheet. Steps should be taken to avoid this.

35 When the surface temperature of the drum dryer is less than 105°C but is 70°C or higher, longer

contact times are required in order for the handsheet to have a final moisture content of 5%.

5 If the surface temperature of the drum dryer is below 70°C, it is necessary to extend the contact time further or increase the initial pressing on the wet mat to remove more water or to do both. It is possible to reduce the moisture content of the pressed sheet to less than 60%.

10

Testing

Conditioning and testing of the paper is done according to procedures laid out in the "Tappi Test Methods" published by TAPPI, Technology Park Atlanta, PO Box 105113, Atlanta GA 15 30348, USA, ISBN 0 - 89852 - 200 - 5 (vol 1 and 2). The wet tensile breaking strength of paper and paper board is defined by method T 456 om - 87; the tensile breaking properties of paper and paper board is T494 om - 81; the HST (Hercules Sizing Test) is defined as size test for paper by ink resistance T 530 20 pm - 83; and the Cobb test is defined by T 441 om - 90.

A series of experiments were performed in which the cellulase concentration, glutaraldehyde concentration, drying time and temperature, aging time and temperature were each varied. The 25 results are presented in the following tables in which:-

"naturally aged" refers to storage for the specified time at 23°C ± 1°C in relative humidity 50.0 ± 2% as specified in T402om-83;

30 "oven cured" refers to treatment at 80°C for 30 minutes;

"standard drying conditions" refers to drying at 105°C for 35 seconds;

The wet and dry tensile strengths were determined by methods 35 T456om-87 and T494om-81, respectively, and the ratio of wet to dry tensile strength expressed as a percentage. These are the data presented in the tables where the higher the value, the better the wet strength. The sizing effect was measured by the

HST (Hercules size test) (TAPPI method T530pm-83) and the data recorded in seconds. The higher the value, the better the sizing. Preferably the HST value is greater than 20g, more preferably greater than 120g, more preferably greater than 5 200g. Size effect was also measured by the Cobb test (TAPPI method T441om-90) and the data recorded in grams/m². "Fully saturated" means that the paper showed no sizing at all. The lower the Cobb value, the better the sizing. Preferably, the Cobb value is less than 30g/m², more preferably less than 10 21g/m².

Wet Strength and Sizing Performance of Cellulase/Glutaraldehyde System in Handsheets dried under standard conditions

15

"% Wet Strength after 24h naturally aged"

| | | Protein on fibre | | |
|----------------------------|-----|------------------|------|------|
| | | 0% | 5% | 10% |
| Glutaraldehyde on fibre | 10% | 0.62 | 1.63 | 2.64 |
| | 20% | 0.80 | 3.44 | 6.24 |
| | 40% | 0.97 | 5.00 | 8.86 |

Control:- 0.25% Kymene SLX = 4.57%

25

"% Wet Strength after 2 weeks naturally aged"

| | | Protein on fibre | | |
|----------------------------|-----|------------------|------|-------|
| | | 0% | 5% | 10% |
| Glutaraldehyde on fibre | 10% | 0.94 | 1.97 | 3.01 |
| | 20% | 0.99 | 3.57 | 6.06 |
| | 40% | 1.05 | 5.56 | 10.30 |

Control:- 0.25% Kymene SLX = 9.01%

35

40

"HST (seconds) after 24h naturally aged"

| | | Protein on fibre | | |
|---|----------------------------|---------------------------------|----|-----|
| | | 0% | 5% | 10% |
| 5 | Glutaraldehyde on fibre | 10% | 1 | 51 |
| | | 20% | 1 | 89 |
| | | 40% | 1 | 108 |
| | | Control:- 0.25% Kymene SLX = 1s | | 159 |

"HST (seconds) after 2 weeks naturally aged"

| | | Protein on fibre | | |
|----|----------------------------|---------------------------------|----|-----|
| | | 0% | 5% | 10% |
| 15 | Glutaraldehyde on fibre | 10% | 1 | 66 |
| | | 20% | 1 | 86 |
| | | 40% | 1 | 120 |
| | | Control:- 0.25% Kymene SLX = 1s | | 132 |

"HST (seconds) after oven curing"

| | | Protein on fibre | | |
|----|----------------------------|---------------------------------|----|-----|
| | | 0% | 5% | 10% |
| 20 | Glutaraldehyde on fibre | 10% | 1 | 60 |
| | | 20% | 1 | 101 |
| | | 40% | 1 | 108 |
| | | Control:- 0.25% Kymene SLX = 1s | | 149 |

25

"Cobb (qsm) after 24h naturally ageing"

| | | Protein on fibre | | |
|----|----------------------------|--|-----------------|------|
| | | 0% | 5% | 10% |
| 30 | Glutaraldehyde on fibre | 10% | Fully saturated | 39.0 |
| | | 20% | Fully saturated | 24.7 |
| | | 40% | Fully saturated | 24.4 |
| | | Control:- 0.25% Kymene SLX = Fully saturated | | 21.6 |

"Cobb (qsm) after 2 weeks naturally ageing"

| | | Protein on fibre | | | |
|---|----------------------------|------------------|-----------------|------|------|
| | | 0% | 5% | 10% | |
| 5 | Glutaraldehyde on fibre | 10% | Fully saturated | 35.3 | 21.1 |
| | | 20% | Fully saturated | 24.1 | 20.4 |
| | | 40% | Fully saturated | 23.1 | 20.3 |

Control:- Kymene SLX = Fully saturated

10 "Cobb (qsm) after oven curing"

| | | Protein on fibre | | | |
|----|----------------------------|------------------|-----------------|------|------|
| | | 0% | 5% | 10% | |
| 15 | Glutaraldehyde on fibre | 10% | Fully saturated | 35.0 | 22.8 |
| | | 20% | Fully saturated | 29.2 | 20.7 |
| | | 40% | Fully saturated | 24.5 | 20.3 |

Control:- 0.25% Kymene SLX = Fully saturated

20 Wet Strength and Sizing Performance on Cellulase/Glutaraldehyde system in handsheets dried under alternative conditions% Wet Strength after 24h naturally aged"

| | | Protein/Glutaraldehyde on fibre | | |
|----|--|---------------------------------|---------|------|
| | | 5%/20% | 10%/40% | |
| 30 | Drying conditions (surface temperature /contact time) | 23°C/ overnight | 0.65 | 4.13 |
| | | 60°C/120s | 2.00 | 5.65 |
| | | 70°C/180s | 2.54 | 6.50 |
| | | 105°C/35s | 2.86 | 6.97 |

Control:- 0.25% Kymene SLX (105°C/35s) = 6.03%

"% Wet Strength after oven curing"

| | | Protein/Glutaraldehyde on fibre | |
|----|--|---------------------------------|---------|
| | | 5%/20% | 10%/40% |
| 5 | 23°C/overnight | 2.14 | 5.66 |
| | 60°C/120s | 3.04 | 6.11 |
| | 70°C/180s | 3.25 | 7.28 |
| | 105°C/35s | 2.99 | 8.82 |
| 10 | Control:- 0.25% Kymene SLX (105°C/35s) | = 11.38% | |

"HST (seconds) after 24h naturally aged"

| | | Protein/Glutaraldehyde on fibre | |
|----|--|---------------------------------|---------|
| | | 5%/20% | 10%/40% |
| 20 | 23°C/overnight | 3 | 3 |
| | 60°C/120s | 51 | 39 |
| | 70°C/180s | 154 | 184 |
| | 105°C/35s | 83 | 208 |
| 25 | Control:- 0.25% Kymene SLX (105°C/35s) | = 1s | |

"HST (seconds) after 2 weeks naturally aged"

| | | Protein/Glutaraldehyde on fibre | |
|----|--|---------------------------------|---------|
| | | 5%/20% | 10%/40% |
| 30 | 23°C/overnight | 3 | 2 |
| | 60°C/120s | 55 | 56 |
| | 70°C/180s | 158 | 221 |
| | 105°C/35s | 82 | 231 |
| 35 | Control:- 0.25% Kymene SLX (105°C/35s) | = 1s | |

"HST (seconds) after oven curing"

| | | Protein/Glutaraldehyde on fibre | |
|----|---|---------------------------------|---------|
| | | 5%/20% | 10%/40% |
| 5 | Drying conditions (surface temperature /contact time) | 23°C/ overnight | 2 |
| | | 60°C/120s | 56 |
| | | 70°C/180s | 134 |
| | | 105°C/35s | 96 |
| 10 | Control:- 0.25% Kymene SLX (105°C/35s) = 1s | | |

15

"Cobb (gsm) after 24h naturally aged"

| | | Protein/Glutaraldehyde on fibre | |
|----|--|---------------------------------|-----------------|
| | | 5%/20% | 10%/40% |
| 20 | Drying conditions (surface temperature /contact time) | 23°C/ overnight | Fully saturated |
| | | 60°C/120s | 26.8 |
| | | 70°C/180s | 21.9 |
| | | 105°C/35s | 28.0 |
| 25 | Control:- 0.25% Kymene SLX (105°C/35s) = Fully saturated | | |

30

"Cobb (gsm) after 2 weeks naturally aged"

| | | Protein/Glutaraldehyde on fibre | |
|----|--|---------------------------------|-----------------|
| | | 5%/20% | 10%/40% |
| 35 | Drying conditions (surface temperature /contact time) | 23°C/ overnight | Fully saturated |
| | | 60°C/120s | 25.6 |
| | | 70°C/180s | 20.4 |
| | | 105°C/35s | 22.7 |
| 40 | Control:- 0.25% Kymene SLX (105°C/35s) = Fully saturated | | |

"Cobb (gsm) after oven curing"

| | | Protein/Glutaraldehyde on fibre | |
|----|--|---------------------------------|-----------------|
| | | 5%/20% | 10%/40% |
| 5 | Drying conditions (surface temperature /contact time) | 23°C/ overnight | Fully saturated |
| | | 60°C/120s | 26.3 |
| | | 70°C/180s | 21.5 |
| | | 105°C/35s | 22.9 |
| 10 | Control:- 0.25% Kymene SLX (105°C/35s) = Fully saturated | | |

15 Wet/Dry Strength and Sizing Performance of Cellulase/Glutaraldehyde system dried under two stage conditions

"% Wet Strength after 48h naturally aged"

| Dry Conditions (Surface temp/contact time) | | | %Wet Strength |
|--|--|-----------|---------------|
| Expt Code | Dryer A | Dryer B | |
| 1 | 40°C/60s | 70°C/180s | 4.66 |
| a | 55°C/60s | 70°C/180s | |
| b | 40°C/180s | 70°C/180s | |
| ab | 55°C/180s | 70°C/180s | |
| c | 40°C/60s | 105°C/35s | |
| ac | 55°C/60s | 105°C/35s | |
| bc | 40°C/180s | 105°C/35s | |
| abc | 55°C/180s | 105°C/35s | |
| 30 | Control:- 0.25% Kymene SLX (105°C/35s) = 8.48% | | |

35 "% Wet Strength after oven curing"

| Dry Conditions (Surface temp/contact time) | | | %Wet Strength |
|--|---|-----------|---------------|
| Expt Code | Dryer A | Dryer B | |
| 1 | 40°C/60s | 70°C/180s | 5.38 |
| a | 55°C/60s | 70°C/180s | |
| b | 40°C/180s | 70°C/180s | |
| 40 | Control:- 0.25% Kymene SLX (105°C/35s) = 5.82 | | |

| | | | | |
|---|-----|-----------|-----------|------|
| | ab | 55°C/180s | 70°C/180s | 2.84 |
| | c | 40°C/60s | 105°C/35s | 5.17 |
| | ac | 55°C/60s | 105°C/35s | 5.49 |
| | bc | 40°C/180s | 105°C/35s | 6.03 |
| 5 | abc | 55°C/180s | 105°C/35s | 5.53 |

Control:- 0.25% Kymene SLX (105°C/35s) = 12.53%

10 "Dry strength after 48h naturally aged"

| Dry Conditions (Surface temp/contact time) | | | Dry Strength /kNm ⁻¹ |
|--|-----------|-----------|---------------------------------|
| Expt Code | Dryer A | Dryer B | |
| 1 | 40°C/60s | 70°C/180s | 5.40 |
| a | 55°C/60s | 70°C/180s | 5.12 |
| b | 40°C/180s | 70°C/180s | 5.43 |
| ab | 55°C/180s | 70°C/180s | 4.77 |
| c | 40°C/60s | 105°C/35s | 5.09 |
| ac | 55°C/60s | 105°C/35s | 5.50 |
| bc | 40°C/180s | 105°C/35s | 5.13 |
| 20 abc | 55°C/180s | 105°C/35s | 5.27 |

Controls:- Blank (105°C/35s) = 4.28kNm⁻¹; 0.25% Kymene SLX (105°C/35s) = 4.41kNm⁻¹

25 "Dry strength after oven curing"

| Dry Conditions (Surface temp/contact time) | | | Dry Strength /kNm ⁻¹ |
|--|-----------|-----------|---------------------------------|
| Expt Code | Dryer A | Dryer B | |
| 1 | 40°C/60s | 70°C/180s | 5.24 |
| a | 55°C/60s | 70°C/180s | 4.65 |
| b | 40°C/180s | 70°C/180s | 5.11 |
| ab | 55°C/180s | 70°C/180s | 4.75 |
| c | 40°C/60s | 105°C/35s | 5.09 |
| ac | 55°C/60s | 105°C/35s | 5.18 |
| bc | 40°C/180s | 105°C/35s | 5.46 |
| 35 abc | 55°C/180s | 105°C/35s | 4.78 |

Controls:- Blank (105°C/35s) = 3.93 kNm⁻¹; 0.25% Kymene SLX (105°C/35s) = 4.64kNm⁻¹

"HST after 48h naturally aged"

| Dry Conditions (Surface temp/contact time) | | | HST / seconds |
|--|---------|-----------|---------------|
| Expt Code | Dryer A | Dryer B | |
| 5 | 1 | 40°C/60s | 70°C/180s |
| | a | 55°C/60s | 70°C/180s |
| | b | 40°C/180s | 70°C/180s |
| | ab | 55°C/180s | 70°C/180s |
| 10 | c | 40°C/60s | 105°C/35s |
| | ac | 55°C/60s | 105°C/35s |
| | bc | 40°C/180s | 105°C/35s |
| | abc | 55°C/180s | 105°C/35s |

Control:- 0.25% Kymene SLX (105°C/35s) = 1s

15

"HST after 2 weeks naturally aged"

| Dry Conditions (Surface temp/contact time) | | | HST / seconds |
|--|---------|-----------|---------------|
| Expt Code | Dryer A | Dryer B | |
| 20 | 1 | 40°C/60s | 70°C/180s |
| | a | 55°C/60s | 70°C/180s |
| | b | 40°C/180s | 70°C/180s |
| | ab | 55°C/180s | 70°C/180s |
| 25 | c | 40°C/60s | 105°C/35s |
| | ac | 55°C/60s | 105°C/35s |
| | bc | 40°C/180s | 105°C/35s |
| | abc | 55°C/180s | 105°C/35s |

Control:- 0.25% Kymene SLX (105°C/35s) = 1s

30

"HST after oven curing"

| Dry Conditions (Surface temp/contact time) | | | HST / seconds |
|--|---------|-----------|---------------|
| Expt Code | Dryer A | Dryer B | |
| 35 | 1 | 40°C/60s | 70°C/180s |
| | a | 55°C/60s | 70°C/180s |
| | b | 40°C/180s | 70°C/180s |
| | ab | 55°C/180s | 70°C/180s |

| | | | |
|-----|-----------|-----------|-----|
| c | 40°C/60s | 105°C/35s | 212 |
| ac | 55°C/60s | 105°C/35s | 275 |
| bc | 40°C/180s | 105°C/35s | 308 |
| abc | 55°C/180s | 105°C/35s | 210 |

5 Control:- 0.25% Kymene SLX (105°C/35s) = 1s

"Cobb after 48h naturally aged"

| 10 | Dry Conditions (Surface temp/contact time) | | | Cobb / gsm |
|-------|--|-----------|---------|------------|
| | Expt Code | Dryer A | Dryer B | |
| 1 | 40°C/60s | 70°C/180s | | 21.7 |
| a | 55°C/60s | 70°C/180s | | 21.9 |
| b | 40°C/180s | 70°C/180s | | 22.0 |
| 15 ab | 55°C/180s | 70°C/180s | | 25.1 |
| c | 40°C/60s | 105°C/35s | | 20.7 |
| ac | 55°C/60s | 105°C/35s | | 20.1 |
| bc | 40°C/180s | 105°C/35s | | 22.1 |
| abc | 55°C/180s | 105°C/35s | | 25.9 |

20 Control:- 0.25% Kymene SLX (105°C/35s) = Fully saturated

"Cobb after 2 weeks naturally aged"

| 25 | Dry Conditions (Surface temp/contact time) | | | Cobb / gsm |
|-------|--|-----------|---------|------------|
| | Expt Code | Dryer A | Dryer B | |
| 1 | 40°C/60s | 70°C/180s | | 20.8 |
| a | 55°C/60s | 70°C/180s | | 19.2 |
| b | 40°C/180s | 70°C/180s | | 28.2 |
| 30 ab | 55°C/180s | 70°C/180s | | 21.2 |
| c | 40°C/60s | 105°C/35s | | 22.1 |
| ac | 55°C/60s | 105°C/35s | | 20.6 |
| bc | 40°C/180s | 105°C/35s | | 21.7 |
| abc | 55°C/180s | 105°C/35s | | 21.9 |

35 Control:- 0.25% Kymene SLX (105°C/35s) = Fully saturated

"Cobb after oven curing"

| Dry Conditions (Surface temp/contact time) | | | Cobb / gsm |
|--|-----------|-----------|------------|
| Expt Code | Dryer A | Dryer B | |
| 5 1 | 40°C/60s | 70°C/180s | 21.7 |
| a | 55°C/60s | 70°C/180s | 20.4 |
| b | 40°C/180s | 70°C/180s | 22.3 |
| ab | 55°C/180s | 70°C/180s | 22.3 |
| c | 40°C/60s | 105°C/35s | 20.5 |
| 10 ac | 55°C/60s | 105°C/35s | 20.4 |
| bc | 40°C/180s | 105°C/35s | 20.9 |
| abc | 55°C/180s | 105°C/35s | 22.3 |

Control:- 0.25% Kymene SLX (105°C/35s) = Fully saturated

15

20 Comparison of the Effect of Different Drying Regimes on Wet Strength and Sizing Performance of Cellulase/Glutaraldehyde System

25 "Effect of Different Drying Regimes on % Wet Strength (oven cured data)"

| | Additive | Drying Conditions (Surface Temp/Contact Time) | | % Wet Strength |
|----|-------------------------------|---|-----------|----------------|
| | | Dryer A | Dryer B | |
| 30 | 5% Protein/20% Glutaraldehyde | 105°C/35s | - | 2.99 |
| | 5% Protein/20% Glutaraldehyde | 70°C/180s | - | 3.25 |
| | 5% Protein/20% Glutaraldehyde | 40°C/60s | 105°C/35s | 5.17 |
| 35 | 5% Protein/20% Glutaraldehyde | 55°C/180s | 70°C/180s | 5.49 |
| | 0.25% Kymene SLX | 105°C/35s | - | 12.53 |

40

"Effect of Different Drying Regimes on HST (oven cured data)"

| | Additive | Drying Conditions (Surface Temp/Contact Time) | | HST / seconds |
|----|-------------------------------|--|-----------|---------------|
| | | Dryer A | Dryer B | |
| 5 | 5% Protein/20% Glutaraldehyde | 105°C/35s | - | 96 |
| | 5% Protein/20% Glutaraldehyde | 70°C/180s | - | 134 |
| | 5% Protein/20% Glutaraldehyde | 40°C/60s | 105°C/35s | 212 |
| 10 | 5% Protein/20% Glutaraldehyde | 55°C/180s | 70°C/180s | 149 |
| | 0.25% Kymene SLX | 105°C/35s | - | 1 |

"Effect of Different Drying Regimes on Cobb (oven cured data)"

| | Additive | Drying Conditions (Surface Temp/Contact Time) | | Cobb / gsm |
|----|-------------------------------|--|-----------|-----------------|
| | | Dryer A | Dryer B | |
| 20 | 5% Protein/20% Glutaraldehyde | 105°C/35s | - | 22.9 |
| | 5% Protein/20% Glutaraldehyde | 70°C/180s | - | 21.5 |
| | 5% Protein/20% Glutaraldehyde | 40°C/60s | 105°C/35s | 20.5 |
| 25 | 5% Protein/20% Glutaraldehyde | 55°C/180s | 70°C/180s | 22.3 |
| | 0.25% Kymene SLX | 105°C/35s | - | Fully saturated |

In conclusion, the above data demonstrates that cellulase/glutaraldehyde treatment of paper leads to improvement in the wet strength, dry strength and sizing of the paper.

11. Demonstration of Bio-metalization of Paper

The bio-metalization of water-leaf paper was demonstrated. The technique was based on the affinity of streptavidin for biotin.

The biotin label was linked to the cellulase which in turn was linked to streptavidin labelled with gold particles.

- Biotinylated cellulase was incubated with the paper pulp in 1/3
5 PBS buffer (pH 7.4), containing Tween 20 (0.1% vv⁻¹) for 45 min at room temperature with shaking. A paper square (6 cm²) was formed, rolled and allowed to dry overnight at ambient temperature.
- 10 Samples of the paper (1.5 cm²) containing the biotinylated cellulase and control samples lacking the biotinylated cellulase, were incubated with 5% (wv⁻¹) BSA in 10 mM PBS pH 7.4, for 30 min at ambient temperature with shaking.
- 15 The Auroprobe BLplus labelled streptavidin conjugate and enhancer (Amersham Ltd., Amersham, U.K.) was used according to the manufacturer's recommendation to attach and visualize the gold particles (Fostel et al., Chromosoma, 90, 254, (1984); Hutchinson et al., J. Cell Biol., 95, 609, (1982)). The
20 enhancer solution coated the gold particles with silver to create an orange/brown colour which was indicative of the presence of the metals. Control sheets which did not contain the biotinylated cellulase did not develop the orange/brown colouration and hence were not coated with the metal.

25

12. Capacitance Measurement of Biometalized Paper

The capacitance of biometalized paper sheets was compared to control sheets to determine if the presence of the gold-
30 labelled cellulase altered the capacitance characteristics of paper.

Paper sheets were produced from W-LP containing either cellulase, gold labelled cellulase, enhanced gold labelled
35 cellulase and cellulase-free controls. The sheets were each held between two metal plates connected to a capacitance meter. The metal plates were held in position in a jig which ensured that a constant and reproducible distance was maintained

between the plates.

The capacitance (C) was calculated using the following equation

5

$$C = \frac{\epsilon_0 \epsilon_r A}{d}$$

where d = distance between the two plates

A = area

10

ϵ_0 = constant

ϵ_r = relative permeability

The measurements obtained indicated an increase in the capacitance of the paper sheets in the presence of gold 15 labelled cellulase. The results of the determination are given in Table 7.

Table 7: Capacitance Determination

| 20 | Sample | Capacitance (pF) |
|----|---------------------------------|------------------|
| | Machine Calibration (control) | 10.00 |
| | Paper without cellulase | 10.97 |
| | Paper + cellulase | 10.65 |
| 25 | Paper + gold labelled cellulase | 13.86 |

13. Demonstration of binding amylase enzymes to starch

Two amylase enzymes were characterized using HPLC: an α -amylase 30 (Type X-A crude preparation) from *Aspergillus oryzae* and amyloglucosidase from *A. niger* (available from Sigma Aldrich Co. Ltd., Poole, Dorset, United Kingdom). The main catalytic peaks of each preparation were determined using a starch glucose-release assay. The binding efficiencies of each 35 protein were determined against a range of starches with BSA controls included in the assessment.

A solution of 32 mg ml⁻¹ (dry weight) of α -amylase was made up

in 0.1 M PBS (pH7.0). 100 μ l of this was loaded onto an HPLC using a Bio-Sil SEC gel permeation column running 0.1 M phosphate buffer at 1 ml min⁻¹. Fractions (1 ml) were collected and tested for reducing sugars released from a starch suspension using the standard microtitre assay (for glucose).

The following qualitative assay was used to detect glucose and cellobiose in test samples. The assay was carried out in a micro titre dish at room temperature.

10

Reagent Components:

- 10 μ l phenol reagent (0.128M phenol in 0.1M phosphate buffer pH7.0)
- 15 10 μ l amino pyrine reagent (19.7mM 4-amino phenazone in 0.1M phosphate buffer pH7.0)
- 10 μ l peroxidase in 0.1M phosphate buffer pH 7.0 (to give 800Eu/ml)
- 10 μ l glucose oxidase in 0.1M phosphate buffer pH 7.0 (to give 20 250Eu/ml)
- 60 μ l 0.1M phosphate buffer pH7.0

These reagent components were mixed and added to the wells of a microtitre dish. Test samples 100 μ l were added followed by 25 an excess of substrate (starch). The appearance of a red colour was indicative of the presence of amylase.

The same methods were also used to produce an HPLC profile for the amyloglucosidase. The amyloglucosidase was a liquid 30 preparation containing approximately 262 mg ml⁻¹ protein as measured by the Coomassie Blue technique. 100 μ l of a 0.007 dilution in 0.1 M PBS (pH 7.0) was loaded onto the HPLC and monitored at 230 nm 0.1 AUS. 1 ml fractions were collected and tested for reducing sugars released from starch suspensions as 35 above.

The ability of α -amylase and amyloglucosidase to bind to normal starch in suspension was assessed. Starch (0.2 g; Roquette)

was added to 9 ml 0.1 M PBS (pH 7.0) and 1 ml α -amylase solution (9.5 mg ml⁻¹ by Coomassie Blue assay) was added. This was incubated on a shaker for 20 min.

- 5 The sample was centrifuged at 13,000 rpm for 5 min and 100 μ l samples loaded onto the HPLC column. The peak profile of the 20 min bound α -amylase was compared with a T = 0 sample. From this data the percentage binding of the enzyme was calculated. The binding of amyloglucosidase was also tested against
10 cationic starch. BSA was also used in the same way as a control. The final concentration of the BSA used was 0.2% (wv⁻¹) in 0.1 M PBS.

The results of the binding experiments are shown in the
15 following Table.

Starch binding profiles

| Enzyme | Substrate | % Bound |
|-------------------|-----------------|---------|
| α -amylase | starch | 32 |
| amyloglucosidase | starch | 27 |
| amyloglucosidase | cationic starch | 45 |
| BSA | starch | 7 |
| BSA | cationic starch | 6 |

25 These results indicate that both α -amylases and amyloglucosidases specifically bind to both starch and cationic starch and are therefore suitable for use as protein linkages for binding effector moieties to starches.

CLAIMS

1. A method of treating a polymer to achieve an improvement in at least one property selected from fluid, electrical and strength properties comprising binding an effector moiety to said polymer via a protein linkage for the purpose of achieving said improvement, said effector moiety being different from said protein linkage and said protein linkage being different from said polymer, said effector moiety and said protein linkage being present in an amount effective to achieve said improvement.
- 15 2. A method of treating a polymer according to claim 1 to achieve an improvement in the fluid penetration properties of said polymer.
- 20 3. A method of treating a polymer according to claim 1 to achieve an improvement in the sizing properties of said polymer.
- 25 4. A method of treating a polymer according to claim 1 to achieve an improvement in the electrical conductivity properties of said polymer.
- 30 5. A method of treating a polymer according to claim 1 to achieve an improvement in the metallic properties of said polymer.
- 35 6. A method of treating a polymer according to claim 1 to achieve an improvement in the wet strength properties of said polymer.

7. A method of treating a polymer according to claim 1 to achieve an improvement in the dry strength properties of said polymer.

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8. A method according to any preceding claim wherein said polymer is a polysaccharide.

10 9. A method according to claim 8 wherein said polymer is cellulose.

10. A method according to any preceding claim wherein said 15 polymer is paper or the constituent fibres of paper.

11. A method according to any preceding claim wherein said 20 protein linkage comprises a naturally occurring enzyme or fragment thereof.

12. A method according to any claim 1 wherein said protein 25 linkage comprises an enzyme selected from the group comprising cellulases, hemi-cellulases, mannases, xylanases, proteinases, keratinases, chitinases, ligninases, agarases, alginases and amylases or fragment thereof.

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13. A method according to any preceding claim wherein said protein linkage comprises a polysaccharidase or fragment thereof.

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14. A method according to claim 13 wherein said protein linkage comprises a cellulase or fragment thereof.

15. A method according to claim 14 wherein said protein linkage comprises a cellulose binding domain of a cellulase.

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16. A method according to any preceding claim wherein said effector moiety is attached to said protein linkage via a linker.

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17. A method according to claim 16 wherein said linker comprises a non-covalent binding pair.

15 18. A method according to any one of claims 1 to 16 wherein said effector moiety is covalently bonded to said protein linkage.

20 19. A method according to any preceding claim wherein said effector moiety is selectively cleavable from said polymer.

25 20. A chemical composition comprising:

a) an effector moiety; and
30 b) a protein capable of binding said effector moiety to a polymer;

wherein said effector moiety is different from said protein and wherein said composition is capable of achieving an improvement in at least one property selected from fluid, electrical and strength properties of said polymer.

35 21. A composition of matter comprising a polymer to which is

bound an effector moiety via a protein linkage, said effector moiety being different from said protein linkage, wherein said effector moiety and said protein linkage are present in an amount effective to achieve an improvement in at least one property selected from fluid, electrical and strength properties of said polymer.

22. A method of treating a polymer to achieve an improvement
in at least one property selected from fluid, electrical
and strength properties comprising contacting said
polymer with an effector moiety and a protein for the
purpose of achieving said improvement, said effector
moiety being different from said protein and also
different from said polymer, and said protein being
different from said polymer, and said effector moiety and
said protein being present in an amount effective to
achieve said improvement.
23. A method according to claim 22 comprising the step of
contacting a conjugate of said effector moiety and said
protein with said polymer.
24. A method according to claim 22 comprising the step of
contacting said effector moiety with a conjugate of said
protein and said polymer.
25. A method of treating a polymer to achieve an improvement
in at least one property selected from fluid, electrical
and strength properties comprising binding at least one
effector moiety to at least one polymer via at least one
protein linkage for the purpose of achieving said
improvement, said at least one effector moiety being
different from said at least one protein linkage and said
at least one protein linkage being different from said at

least one polymer, said at least one effector moiety and said at least one protein linkage being present in an amount effective to achieve said improvement.

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26. A method of treating a polymer to achieve an improvement in at least one property selected from fluid, electrical and strength properties comprising contacting at least one polymer with at least one effector moiety and at least one protein for the purpose of achieving said improvement, said at least one effector moiety being different from said at least one protein and also different from said at least one polymer, and said at least one protein being different from said at least one polymer, and said at least one effector moiety and said at least one protein being present in an amount effective to achieve said improvement.
- 20 27. A method of treating paper or the constituent fibres of paper to achieve an improvement in at least one property selected from fluid, electrical and strength properties comprising binding at least one effector moiety to said paper or constituent fibres of paper via at least one protein linkage for the purpose of achieving said improvement, said at least one effector moiety being different from said at least one protein linkage and said at least one protein linkage being different from said paper or constituent fibres of paper, and said at least one effector moiety and said at least one protein linkage being present in an amount effective to achieve said improvement.
- 25 30
- 35 28. A method of treating paper or the constituent fibres of paper to achieve an improvement in at least one property selected from fluid, electrical and strength properties comprising binding an effector moiety to said paper or

5 constituent fibres of paper via a protein linkage for the purpose of achieving said improvement, said effector moiety being different from said protein linkage and said protein linkage being different from said paper or constituent fibres of paper, and effector moiety and said protein linkage being present in an amount effective to achieve said improvement.

- 10 29. A method according to claim 28 wherein said effector moiety is capable of conferring improved wet strength on said paper.
- 15 30. A method according to claim 28 wherein said effector moiety is capable of conferring improved dry strength on said paper.
- 20 31. A method according to claim 28 wherein said effector moiety is capable of conferring improved sizing on said paper.
- 25 32. A method according to claim 28 wherein said effector moiety is a cross-linking agent.
- 30 33. A method according to claim 32 wherein said effector moiety is a dialdehyde cross-linking agent.
- 35 34. A method according to claim 33 wherein said effector moiety is glutaraldehyde.
35. A method according to any one of claims 28 to 34 wherein said protein linkage is a cellulase.

36. Use of an effector moiety and a protein in a method of
treating a polymer to achieve an improvement in at least
one property selected from fluid, electrical and strength
5 properties of said polymer.

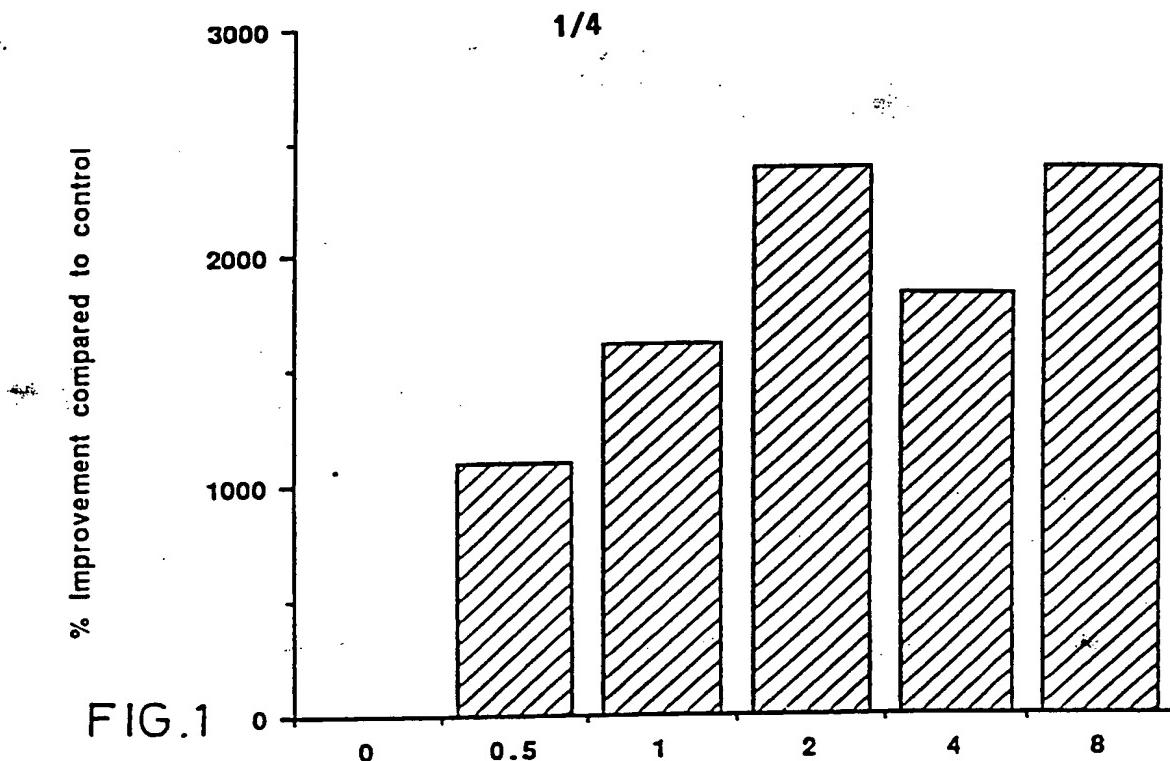


FIG. 1

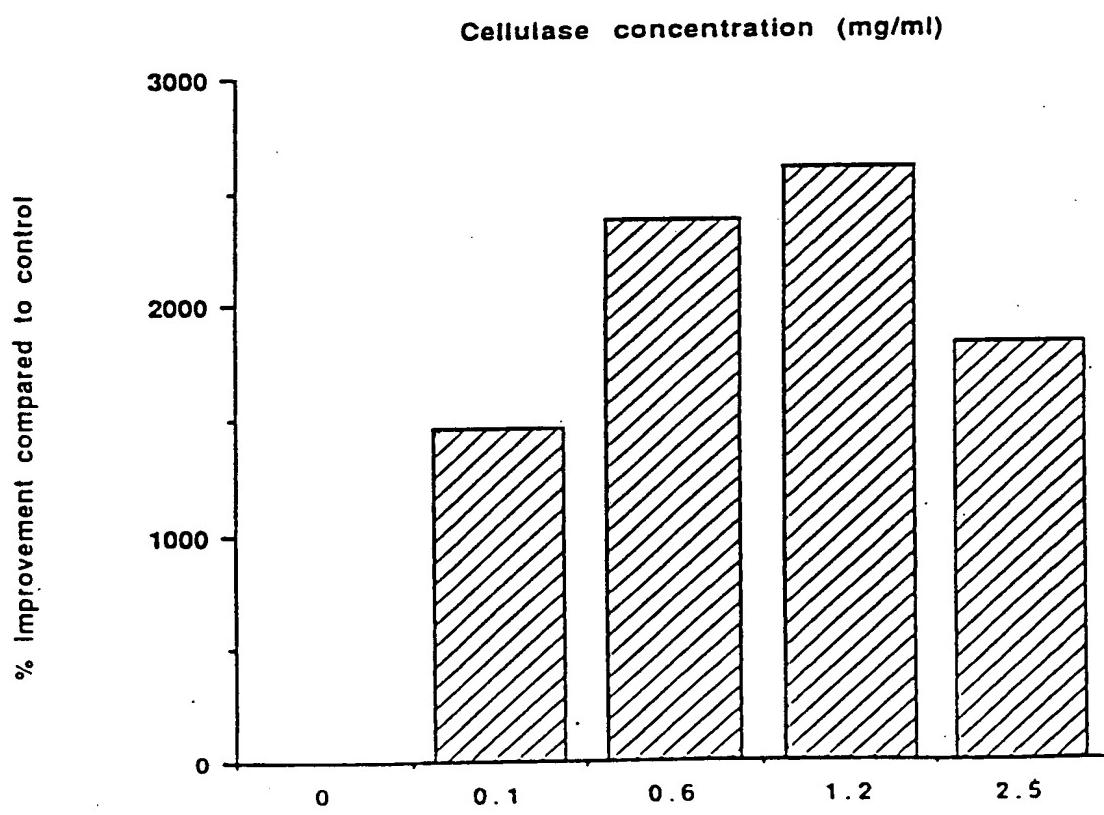
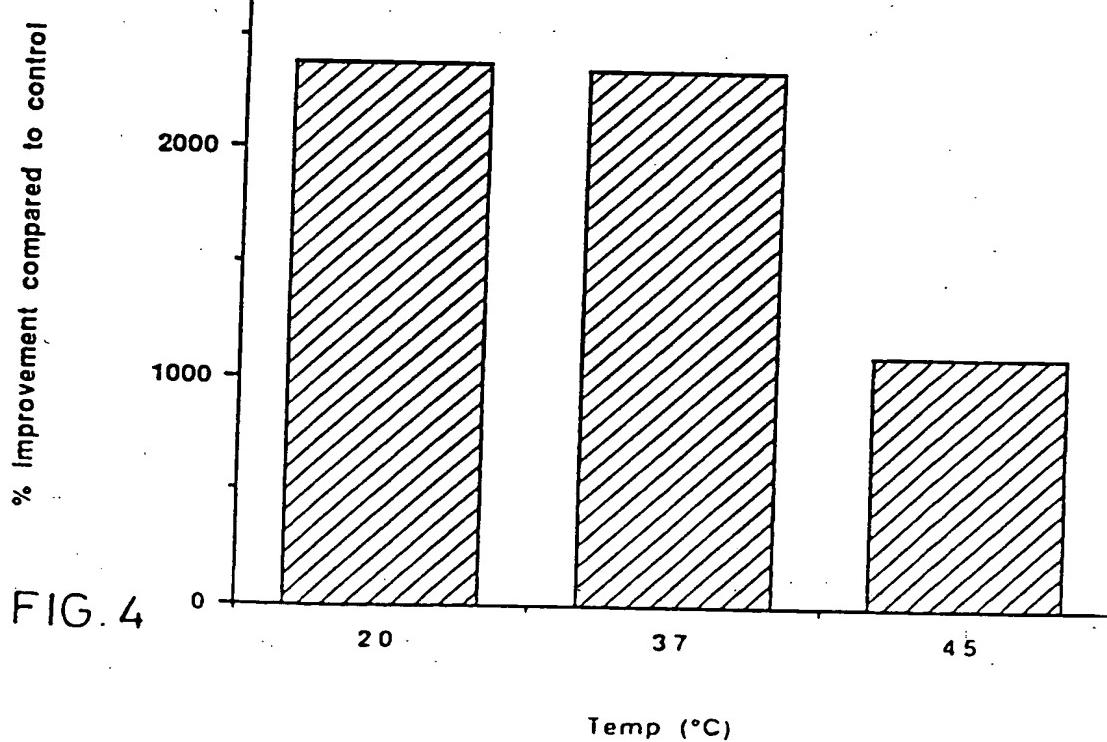
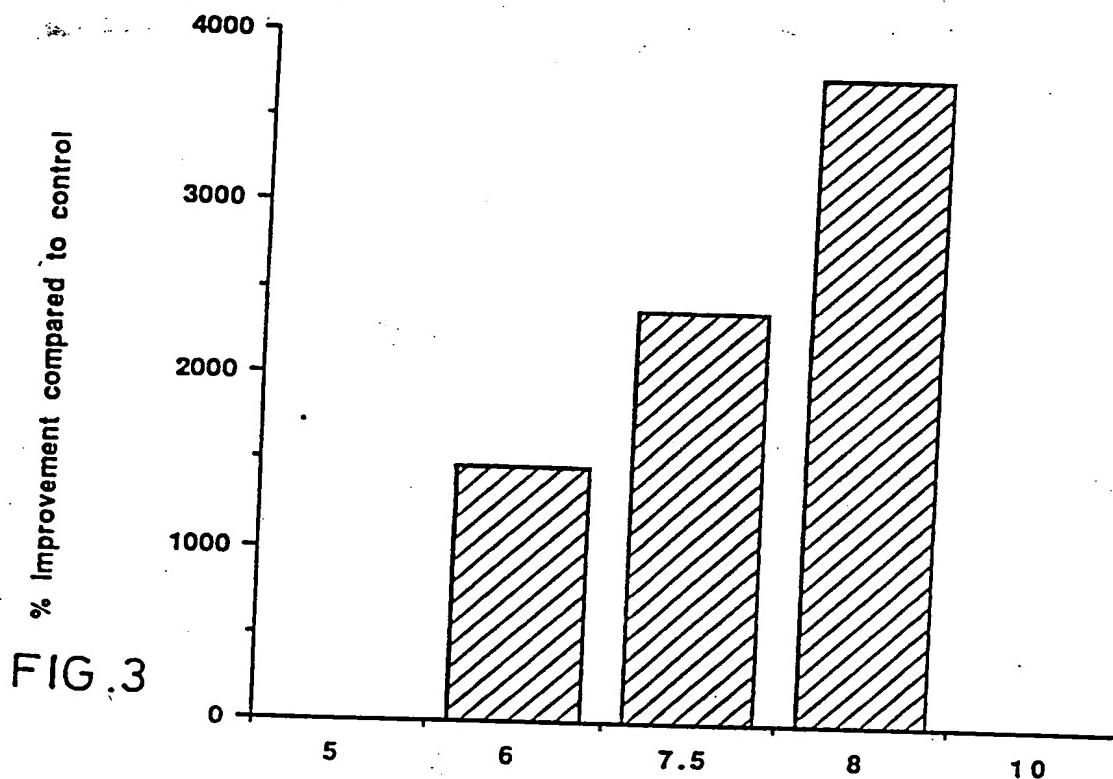
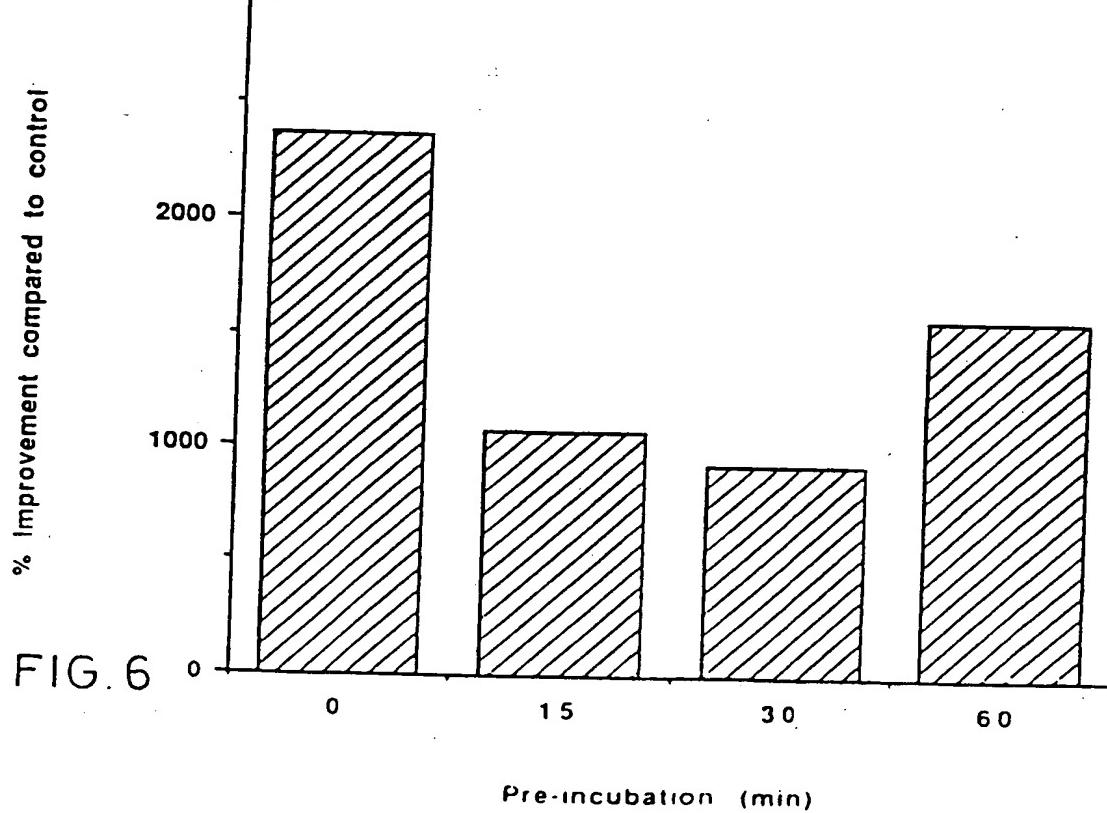
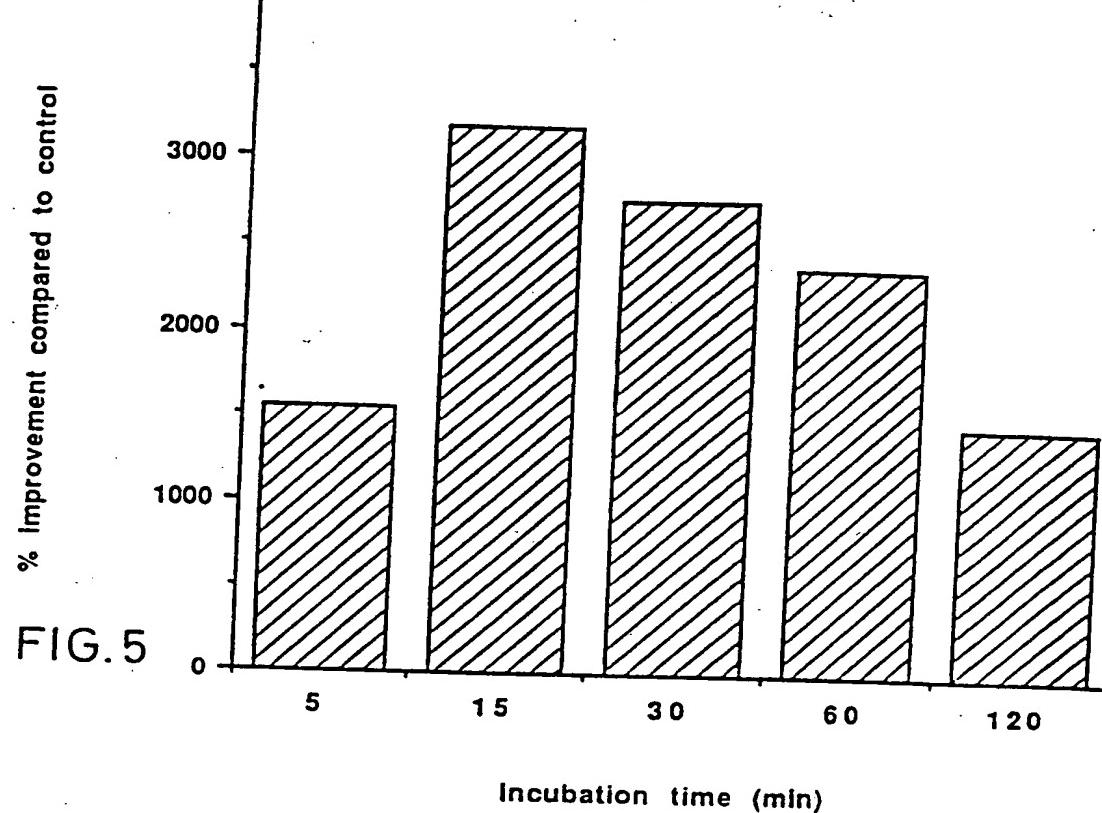


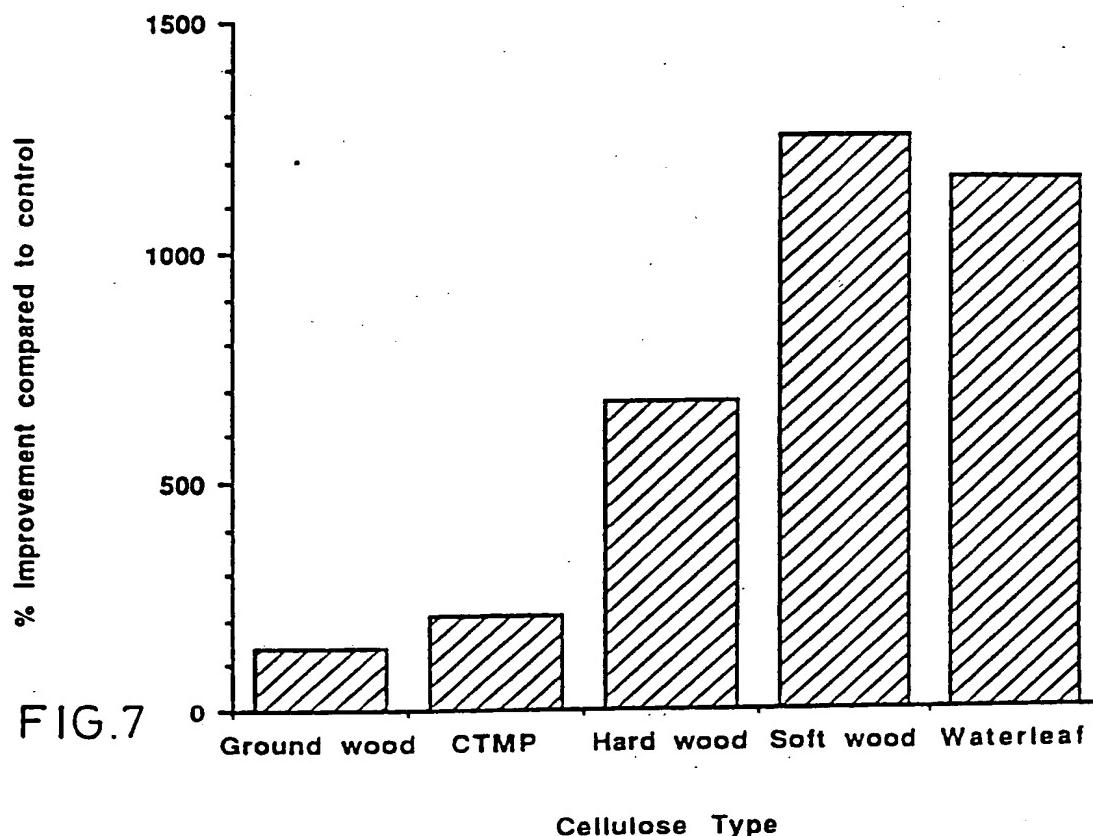
FIG. 2

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**SUBSTITUTE SHEET (RULE 26)**



INTERNATIONAL SEARCH REPORT

Inte... al Application No
PCT/GB 96/02009

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N11/12 D21H17/22 D21H21/14

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 D21H C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|------------|---|-----------------------|
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| X | WO 93 05226 A (UNIV BRITISH COLUMBIA) 18 March 1993 cited in the application see the whole document --- | 1-36 |
| A | FR 2 204 633 A (AMERICAN CYANAMID CO) 24 May 1974 see the whole document ----- | 1-36 |

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

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Date of the actual completion of the international search

22 November 1996

Date of mailing of the international search report

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 96/02009

| Patent document cited in search report | Publication date | Patent family member(s) | | Publication date |
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